

Abstract

Title of dissertation: CHITINASE-AN EVOLUTIONARY DUALITY

Qiong Tang, Doctor of Philosophy, 2008

Dissertation directed by: Professor Allen R. Place

Marine, Estuarine and Environmental Sciences Program

The purpose of this thesis was to characterize the molecular properties, gene copy number and gene organization for the chitinase enzyme in a lower vertebrate. Rainbow trout (*Oncorhynchus mykiss*) has one of the highest chitinase activities in all the fishes examined. I also aimed to clarify the evolutionary origin and associated regulatory control of the chitinase gene in this species.

The cDNAs from two chitinases (Onmy-Chit.01 and Onmy-Chit.02) were successfully cloned and characterized from rainbow trout tissues. Onmy-Chit.01 is predominately expressed in the stomach, with high mRNA expression in the gastric gland. Its protein secretes along mucosal folds to the stomach lumen. Onmy-Chit.02 is primarily expressed in immunity related organs, such as kidney, spleen, and liver, as well as in reproductive organs. From *in situ* hybridization and flow cytometry analysis, I show that Onmy-Chit.02 is constitutively expressed in the myeloid cell lineage of the rainbow trout immune system. These two enzymes share many similarities with their mammalian orthologs. Their predicted proteins all have classic chitinase protein structures. In addition, they all have O-glycosylation sites but with different pH optimas.

Fluorescent *in situ* hybridization (FISH) shows that both chitinases are located on Chromosome 17 of the rainbow trout genome. Upon full sequencing of two BACs

containing Onmy-Chit.01 or .02, I found two copies of Onmy-Chit.01 with almost identical coding regions, but with very different promoter regions. The two copies are approximately 9Kb apart and sit in a head to tail arrangement. Only one copy of Onmy-Chit.02 was found in the rainbow trout genome, and its distinct promoter regions are distinct from both copies of Onmy-Chit.01.

Phylogenetic analyses revealed that the chitinase gene family fits an evolutionary birth and death model with the chitinase genes derived by duplication of an ancestral chitinase gene. Further gene duplication and loss of chitinolytic activity in mammals gave rise to chitolectins.

Hence, I postulate that the function of chitinase is two fold: 1) it is a key element in the first line defense of the innate immunity repertoire; and 2) it serves as a gastric digestive enzyme for chitin containing food items.

CHITINASE-AN EVOLUTIONARY DUALITY

By

Qiong Tang

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Advisory Committee:
Professor Allen R. Place, Chair
Professor Martin F. Flajnik
Professor John D. Hansen
Professor Renate Reimschuessel
Professor John M. Trant
Professor Thomas D. Kocher

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List of Abbreviations

AMCase	Acidic Mammalian Chitinase
CHI3L1	Chitinase 3-like 1, also known as YKL40 or HC-gp39
CHI3L2	Chitinase 3-like 2, also known as YKL39
CHIA	Acidic Mammalian Chitinase Gene
CHIT1	Chitotrisidase Gene
ECR	Evolutionary Conservative Region
IFN- γ	Interferon-- γ
IHC	Immunohistochemistry
IL-13	Interleukin 13
IL-4	Interleukin 4
IL-5	Interleukin 5
ISH	<i>in situ</i> hybridization
LAMP	lysosomal-associated membrane proteins
LPS	Lipopolysaccharide
MPR	mannose 6-phosphate receptors
NAG	N-acetylglucosamine
RACE	rapid amplification of cDNA ends
TFBS	Transcription Factor Binding Site
Th1	Helper T cells type 1
Th2	Helper T cells type 2
TLR	Toll Like Receptor
TNF- α	Tumor Necrosis Factor- α

Chapter I General Introduction

I.A. Chitin and Chitosan

Chitin is an unbranched homopolymer of β -(1, 4) linked N-acetyl glucosamine (NAG) residues and is the second most abundant carbon polymer, after cellulose, on earth [1]. It is especially important in the aquatic environment due to the abundance of chitin-containing invertebrates. Although chitin was isolated for the first time from mushrooms by Braconnot in 1811, about 30 years earlier than cellulose, it remained an unused industrial biomass resource for a long time, in sharp contrast to cellulose. However, interest in this abundant biopolymer has increased significantly in recent years.

Three forms of chitin are known to exist. In the most common form, α -chitin, the adjacent polymer chains run in opposite directions, an anti-parallel arrangement. This form of chitin is particularly stable because the anti-parallel arrangement allows not only for inter-chain hydrogen bonding, but also intramolecular hydrogen bonding. A less common form of chitin is β -chitin, in which the N-acetylglucosamine chains are parallel to one another and form no inter-sheet hydrogen bonds. This arrangement of polymer chains is more flexible than that of α -chitin, but is still quite strong. β -chitin can be obtained from squid pens, and also existed in *Aprodite chaetae*, lorica of sessile ciliate, pogonophore tubes, and diatom spines. The third form of chitin is γ -chitin, in which the polymer chains have mixed parallel and anti-parallel orientation [2]. The idealized structure of chitin, alpha, beta or gamma, is completely acetylated; in fact, though, at least one in six residues in any given chitinous structure will be de-acetylated. The

completely de-acetylated form of chitin is called chitosan and is found naturally occurring only in some fungi, which is believed to be a product of chitin-deacetylases.

Due to their naturally non-toxic, biocompatible, and biodegradable properties, both chitin and chitosan derivative are used as a support polymer for drug, DNA and vaccine carriers to improve the transport of these bioactive molecules through mucosae and epithelia[3], and they can also be expected as a useful excipient to give rise to controlled-release[4]. Chitosan has also been used in the nutritional supplement industry; it was made available over the counter as a dietary supplement or cholesterol-lowering agent. The mechanism of this effect was thought to be that the positively charged chitosan binds to free fatty acids and bile salt components and hence inhibits lipid absorption[5].

In medical practice, non-woven chitin fabrics and chitin threads are used as artificial skins and sutures with the advantages of biocompatibility and biodegradability in wound healing and as an oral mucosal adhesive. Chitin and chitosan evidently promote wound healing and enable high quality cosmetic restoration. Because of the remarkable hemostatic activity, chitosan bandages are being used by paramedics in the U.S. forces in Iraq and Afghanistan to stop bleeding instantaneously. The effect of chitin/chitosan mixture membrane on burn wound healing depends on the deacetylation degree of chitosan, and chitosan with a degree of deacetylation of 96% provided the greatest reduction in the wound area among all the chitosan tested[5].

I.A.1. Chitin in marine environment

Chitin is one of the most abundant carbohydrates present in the marine environment. Annual biosynthesis estimates range from 10^{10} to 10^{11} metric tons[6]. Crustacean exoskeletons are the most common chitinous structures and also the most common

resources to extract chitin. In 2000, the worldwide production of chitin was estimated to reach 10,000 tons, which primarily reflects extraction from the shells of marine crustaceans. Shrimp and crab shells are convenient waste from the seafood processing industry, and contain 15~40% of α -chitin. Chitin is not soluble in water or ordinary solvents and it is fairly stable in mild acidic and basic conditions. Thus, shell treatments typically involve mild acid to remove metal salts, primarily calcium carbonate, followed by mild alkaline treatment several times to remove most of the proteins and pigments. After drying, the product is a colorless to off-white flake or powdery α -chitin. Practically, chitin is used only as a raw material for the production of chitosan, oligosaccharides, and glucosamine [7]. In addition, chitin is also an important structural element for many marine pathogenic organisms, primarily fungi and nematodes.

I.A.1.1. Fungal disease of fish

The fungi are members of the Thallophyta, or non-vascular plants, which are distinguished from the Algae, principally by their complete lack of chlorophyll. They are responsible for a range of serious and economically important diseases of teleost fish [8]. Generally, fungal infections in fish are considered secondary to some other factors or pathogens, a consequence of water quality problems, poor condition, trauma (rough handling or aggression), bacterial disease, or parasites. To some extent, susceptibility to the fungal diseases has significant implications for the fish farming industry in Europe, especially with regard to the rate at which the disease is spreading [9].

Two major groups of fish pathogenic fungi are *Saprolegnia* and *Ichthyophonus*. Interestingly, the *Saprolegnia* cell wall is comprised of cellulose and glycans, and not chitin[10], while the cell wall of *Ichthyophonus* does contain chitin[11].

I.A.1.2. Nematode diseases of fish

Chitin is a component of the eggshells of many parasitic nematodes, and it has also been detected in the feeding apparatus of the strongyloid nematode *Oesophagostomum dentatum* [12], and in the pharyngeal lumen walls of adult and larvae of *Candida elegans* [13].

In commercial fisheries, nematodes infect many different species of aquacultured based and wild fish. Small numbers of nematodes often occur in healthy fish, but high numbers cause illness or even death. In aquaculture systems, brood stock infected with a small number of nematodes may not even show signs of illness, but they often have reduced reproductive capacity. On the other hand, juvenile fish infected by small numbers of nematodes are more likely to show signs of illness and also have reduced growth rates.

In aquaculture situations, fish become infected with nematodes if they are fed live foods containing infective life stages or if they are raised in culture settings that promote the growth of other animals that carry the infective stages of the nematode (vector or paratenic host) or allow nematodes to complete their life cycle (intermediate hosts). Some nematodes can be transmitted directly from fish to fish.

Adult nematodes are typically found in fish digestive tracts. However, depending upon the species of nematode and the species of infected fish, adult and other life stages of nematodes can be found in almost any part of the fish, including the coelomic (body) cavity, internal organs, the swim bladder, deeper layers of the skin or fins, and external muscle layers.

Representative genera of nematodes in aquaculture are *Capillaria*, *Eustrongylides*, *Camallanus*, *Contracaecum*, of which all contain chitin in their eggshells.

I.A.2. Chitin as an immune stimulant

Despite its abundance and wide distribution in pathogenic organisms, chitin was not recognized as an immunostimulant until 1982 [14]. Despite numerous studies using zymosan, a cell wall extraction of fungi, as an immunostimulant, it was thought that β -glucan was the active ingredient of zymosan for priming the immune system, but recently, the importance of chitin in this role has been recognized [15].

I.A.2.1. Mammalian System

Okawa et al. found that both chitin and chitosan could boost host immune systems in fungi-infected mice[16]. Mice pretreated with chitin and chitosan showed resistance to both *Pseudomonas aeruginosa* and *Listeria monocytogenes* after intravenous and intraperitoneal infections. They concluded that the chitin- and chitosan-induced increase in bactericidal activity is primarily due to the induction of polymorphonuclear leucocytes (PMN) and macrophages[16].

Phagocytosable chitin (1-10 μ m) could activate the alveolar macrophage and spleen cell, which produced the IL-12, TNF- α and IL-18[17, 18]. All of these cytokines stimulate the NK cells to produce IFN- γ . IL-10 has an inhibitory effect on chitin priming of alveolar macrophage[19]. Chitin acts as a Th1 adjuvant to up-regulate the Th1 immunity but down-regulate Th2 immunity against a mycobacterial protein (MPB-59) in both IL-10 knockout and wild-type mice. Th2 cells are recognized by their secretion of IL-4, IL-5, IL-9 and IL-13, as opposed to Th1 cells, which secrete IL-2 and interferon- γ ,

although the clear distinction between Th1 and Th2 cells is not as distinct in humans as in mice.

The magnitude of these Th1 adjuvant effects was greater in IL-10-knock out mice than in wildtype [20]. They emphasized that the chitin particulate has to be within 1-10µm size range, since neither soluble chitin oligosaccharides (presumably less than 6 oligomers) nor chitin particulate (50-100µm) are able to prime the alveolar macrophage. Phagocytosable chitosan also primes alveolar macrophages but at a significantly lower level for the elicited oxidative response than the phagocytosable chitin. They claim that initial phagocytosis of chitin particles or bacteria are the key event for alveolar macrophage priming[18].

When chitin was given as oral intake to elderly volunteers (age range, 74-86 years; mean, 80 +/- 3 years), interleukin (IL)-12 and interferon γ levels were significantly higher in the chitin-intake group than in the control group ($P < .05$). However, levels of the inflammatory cytokines IL-1 β and tumor necrosis factor- α decreased after chitin intake [21]. The results of this study suggest that the oral intake of chitin may have beneficial effects on specific cell-mediated immunity while also acting as an anti-inflammatory agent in aged human subjects.

I.A.2.2. Fish system

Sakai et al. [22] reported that rainbow trout injected with chitin exhibit macrophage activities with an increased resistance to *V. anguillarum* infection. Yellowtail injected with chitin alone also showed an increased protection against a *P. piscicida* challenge that continued until 45 days after treatment. Despite the increase in non-specific protective

immunity in yellowtail, chitin did not act as an adjuvant [23], as demonstrated in mice and guinea pigs [24].

The impact of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) was examined by feeding fish diets containing no (control), 25, 50 or 100 mg/kg chitin for 2, 4 and 6 weeks [25]. Lysozyme and natural haemolytic complement activities, together with head-kidney leucocyte respiratory burst, phagocytic and cytotoxic activities, were studied. Lysozyme activity was unaffected by the administration of chitin. The innate humoral and cellular immune response activities were enhanced by the dietary intake of chitin, each activity showing increases at different times - natural haemolytic complement activity and cytotoxic activity after 2 weeks of treatment, respiratory burst activity from 4 weeks and phagocytic activity after 6 weeks. Further studies showed that yeast supplements enhanced phagocytic, respiratory burst, myeloperoxidase and natural cytotoxic activities of head-kidney leucocytes, but not serum complement titres as the humoral response [26]. The enhancement was dose-dependent except for the cytotoxic activity that was only stimulated by the lower dose of yeast assayed.

Recently, the same group reported [27] that gilthead seabream head-kidney leucocytes were able to phagocytose chitin particles smaller than 10 μ M. However, in contrast to the data for mammals, in which large particles (>10 μ M) failed to increase the immune system status, they show that non-phagocytosable chitin particles (>10 μ M) could equally stimulate the seabream immune system. Base on this finding, it is very possible that fish chitinase are able to degrade the large chitin particles to phagocytosable size. They also test the natural cytotoxic activity of nonspecific cytotoxic cells (NCC) upon the incubation with chitin particles, the natural cytotoxic activity particles increased

after incubation for 1 or 4 h, while reduced after incubation for 24 or 48 h in a dose dependent manner. More important, the activity increase differed for each one of the chitin fractions used, maximum activity being reached when particle size $>10\mu\text{M}$ was assayed [28].

I.B. Chitinase Classification and Structure

Production of chitin in the Earth's biosphere ranges in the billions of tons annually. In spite of the stability, insolubility and abundance of chitin, no accumulation of this polymer is observed in the global nutrient cycles. It is believed that chitin is readily recycled enzymatically, thus preventing depletion in global carbon and nitrogen. It has been proposed that if chitin were not recycled in the environment, carbon and nitrogen would be depleted in a matter of decades[29]. There are multiple potential pathways for the breakdown of chitin initiated by distinct enzymes and resulting in distinct products, but eukaryotes mainly uses chitinases to digest chitin, which hydrolyzing the β -(1 \rightarrow 4) glycosidic bonds of the chitin molecule resulting in chitin oligomers.

I.B.1. Classification

Chitinases are given the IUB nomenclature E.C 3.2.1.14, which is based on the type of reaction that the enzyme catalyzes and on substrate specificity. E.C. stands for Enzyme Commission; the first three digits indicate that this enzyme hydrolyses *O*-glycosyl linkages; the last number indicates the substrate and reflects the molecular mechanism. Thus, a chitinase can be defined as “an enzyme catalyzing random hydrolysis of N-acetyl- β -D-glucosaminide 1,4- β -linkages in chitin and in chitooligomers” [30]. However,

this nomenclature does not distinguish between the varying mechanisms of enzymatic chitin hydrolysis.

Henrissat proposed a new classification system for glycoside hydrolases, which was based on similarities in amino acid sequence rather than on substrate specificity[31]. Because of the direct relationship between sequence and folding properties, this classification also reflects structural similarities and, hence, reaction mechanism. Hydrolysis can give rise to either an overall retention (retaining enzymes) or an inversion (inverting enzymes) of anomeric configuration of the substrate. Hydrolysis also produces varying products of endo- and exo- chitinases. According to Henrissat's classification, there are two families of chitinases: glycosyl hydrolase Families 18 and 19. All chitinases with the exception of many plant chitinases and bacterial chitinases are grouped together in Family 18 of the glycoside superfamily.

I.B.2. Structure of Family 18 chitinase

Four family 18 chitinases have been crystallized and structurally analyzed[28, 32, 33]. Generally, family 18 chitinases (Figure I-2) contain two major functional domains: a catalytic domain and a chitin-binding domain; and two additional regions: a signal peptide and a linker region.

I.B.2.1. Catalytic Domain

The catalytic domain of Family 18 chitinase has a TIM-barrel fold. That is, eight strands of parallel β sheet are laid down with a α helix as the "return stroke". The eight strands of the sheet bend into a barrel structure with the helices forming a ring toward the outside[34]. Furthermore, the main active site is a conserved DxxDxDxE region at the

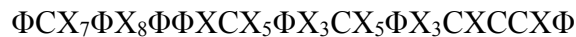
end of the Strand 4. Mutation of the last aspartate and glutamate will cause the loss of activity because they are responsible for protonation of the glycosidic bond[35]. Besides this catalytic core, there are several other essential conserved sites such as a tryptophan at the end of β 8 strand. This tryptophan serves as an “anvil” onto which the –1 sugar is pressed while specific hydrogen bonds with other residues may force the sugar into the boat conformation required for the attack of the N-acetyl group on the anomeric carbon. Thus, deletion of this tryptophan could in itself lead to a completely inactive enzyme.

I.B.2.2. Chitin-Binding Domain

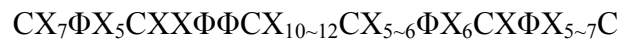
The chitin binding-domain (Figure I-3) is a protein domain that can recognize N-acetylglucosamine or N-linked oligosaccharides on glycoproteins. Besides chitinase, chitin-binding domains exist in numerous proteins such as lectins, VCBP, etc. They all share some structural similarities: conserved cysteine residues that form three to four disulfide bonds, and conserved aromatic residues that form hydrophobic grooves for substrate binding. The typical structure of CBD is formed by three to four antiparallel β -strands connected by long loops and has several surface exposed aromatic residues[28].

The conserved region varies among CBDs from different origins:

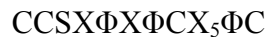
Vertebrate chitinases' chitin-binding domain:



Amphioxus VCBP:



Plant chitinases' CBD:



C represents conserved cysteine residues; Φ represents an aromatic residue, either Phe (F), Tyr (Y), or Trp (W); X represents a non-conserved position, subscript number represents the number of residues.

The vertebrate chitin-binding domain (ChBD) has been classified (by sequence) as a family XII ChBD and exhibits sequence similarity with family V cellulose-binding domains (CeBDs) [36].

Most of chitinase have a single chitin-binding domains but in some chitinases there are two or more, such as in *Brassica juncea* chitinase BjCHI1 [37].

All known vertebrate chitinase's chitin-binding domains are located on the C-terminal end, and the minimum functional distance of the chitin-binding domain from the C-terminus is 49 amino acids. Deletion of as few as two amino acids completely abolishes binding activity [38].

I.B.2.3. Linker Region

The chitin-binding domain is connected to the catalytic domain by a linker region. This linker starts at the last helix of the TIM barrel core, and ends at approximately the first residue of the chitin-binding domain. Structural analysis reveals that this linker runs along the surface of the catalytic domain and has no defined secondary structure. Linkers between catalytic and substrate-binding domains in glycosyl hydrolases have been suggested as being flexible, however this seems to not always be the case [39].

Evidence that the linker region stabilizes the enzyme comes from a study of insect chitinases in which they compare recombinant proteins with and without linker regions and shows that peptides with a linker region are much more stable in the presence of

midgut proteases. Apparently, the linker region protects protease-susceptible bonds in the catalytic domain from hydrolysis in the gut[40].

Peptides linking protein domains are very common in nature and many are believed to join domains rather passively without disturbing their function or affecting their susceptibility to cleavage by host proteases. Linker peptides with G,T or S residues are most common, perhaps because those residues are relatively small with G providing flexibility and T and S being uncharged but polar enough to interact with solvent or by their ability to hydrogen bond to water or the protein backbone to achieve conformational and energetic stability.

I.C. Chitinases in Vertebrates

For a long time it was believed that vertebrates do not have chitinase and that the chitinolytic activity that had been observed in the gastrointestinal system of vertebrates came from lysozyme. In 1994 when Rolf Boot et al. purified and cloned the first vertebrate chitinase[41, 42], which was accidentally named as chitotriosidase.

The Place lab has been working on vertebrate chitinases for 15 years. I examined gastrointestinal tissues from every class of vertebrate species (chondrichthyes, osteichthyes, amphibians, reptiles, birds, and mammals) by a radiometric enzyme assay and they all possess a homologous chitinase. Chitinase activity assays were conducted at pH 2.0, 4.5 and 7.5. All tissues that exhibited chitinase activity showed the highest activity at pH 2 with the exception of the *Xenopus laevis* pancreas, which had a pH optimum of 4.5. Hydrolytic products of chitin were analyzed by Fluorophore Assisted Carbohydrate Electrophoresis (FACE). The primary product of chitin degradation by vertebrate crude extracts of gastric tissues is chitobiose, the dimer of N-acetylglucosamine (NAG).

In addition, reptiles, amphibians and birds (alligator, *Xenopus laevis*, kestrel, dove prion, and Leach's storm petrel) show evidence of a pancreatic chitinase that has characteristics that distinguish it from the gastric enzyme. Not all species showed evidence of gastric chitinase biosynthesis; bluefish, zebrafish, tilapia, and gizzard shad gastric tissues had very low chitinase activity and were devoid of crossreactivity with the GCase antibody.

I.C.1. Mammalian Chitinases

Two kinds of chitinases have been cloned and characterized extensively in human and mouse model.

I.C.1.1. Chitotriosidase

Not until the middle of 1990's was the first mammalian chitinase, chitotriosidase, purified and cloned. This chitinolytically-active protein was first observed in macrophages of human patients with Gaucher's Syndrome [43]. The name "chitotriosidase" was given because the artificial substrate 4MU-chitotriose (4-methylumbelliferyl β -D-N, N', N''-triacetylchitotriose) was first used to identify active fractions [41]. Gaucher's disease is an autosomal recessive inherited lysosomal disorder. In Gaucher's disease patients, plasma chitotriosidase is elevated a thousand times.

In other inherited lysosomal storage disorders a more modest elevation of chitotriosidase was observed, such as in Niemann Pick[44], GM1-gangliosidosis[45], and Krabbe's disease[46]. Also, plasma chitotriosidase expression levels are increased in other diseases too, such as atherosclerosis[47], malaria[48], acute ischemic stroke[49], and thalassemia[50].

Chitotriosidase mRNA is expressed only at later stages of differentiation of monocytes to activated macrophages in culture[51]. Chitotriosidase is also found in the granules of neutrophilic granulocytes [52], and interestingly, neutrophils secrete chitotriosidase upon stimulation of calcium with ionomycin or N-formyl-methionyl-leucyl-phenylalanine (fMLP) [53].

Chitotriosidase is synthesized as a 50-kDa protein with a pI of about 6.5 to 7.2. It is predominantly secreted, but also in part processed into a 39-kDa form with a pI of 8.0 that accumulates in lysosomes. In the C-terminal extension of the 50-kDa chitotriosidase sialic-acid containing O-linked glycans are present, causing its heterogeneous acidic isoelectric point. Chitotriosidase lacks N-linked glycans and the mechanism of routing to lysosomes proves to be distinct from that of soluble, N-glycosylated, lysosomal enzymes. It was observed that, in macrophages, alternative splicing generates a distinct chitotriosidase mRNA species encoding a 40-kDa chitotriosidase that is C-terminally truncated. This enzyme is almost identical to the 39-kDa chitotriosidase formed from the 50-kDa precursor by proteolytic processing. It was concluded that the C-terminus present in the 50-kDa chitotriosidase, but absent in the 39-kDa isoform, was found to mediate tight binding to chitin. In the blood stream the secretory 50-kDa chitotriosidase occurs predominantly, whilst in tissues the 39-kDa form is also abundant[54].

Chitotriosidase is not a house-keeping enzyme[51] and macrophages are able to produce large amounts of this enzyme under specific circumstances, such as in the prolonged culture of macrophages derived from peripheral blood monocytes[51]. On some occasions it represents approximately 1% of the total protein secretion[51].

Chitotriosidase polymorphism

In human, a recessive inherited deficiency of chitotriosidase is frequently encountered in different ethnic groups[55]. A 24-bp duplication in exon 10, where a cryptic splice site is located, generates an abnormally spliced mRNA with an in-frame deletion of 87 nucleotides. The spliced mRNA encodes an enzymatically inactive protein that lacks an internal stretch of 29 amino acids[56]. This allelic polymorphism occurs in certain ethnic groups. For example in Ashkenazi Jewish and Dutch populations, 33-35% of people carry this mutant allele, and 6% are homozygous[56]. In a Portuguese population, approximately 6% and 40% of the population are homozygous and heterozygous respectively[57]. In the Mediterranean region the heterozygote frequencies are 44–54% in Sicily and 32.71% in Sardinia, whereas corresponding values for homozygous Chitotriosidase deficiency were 5.45% and 3.73%, respectively. In contrast, in African subjects from Benin and Burkina Faso a low incidence of chitotriosidase mutation was found (heterozygous 0% and 2%, respectively) and no subject was homozygous for chitotriosidase deficiency[58]. The multi-ethnic occurrence and prevalence of chitotriosidase gene mutation suggests that the enzyme is redundant in humans and that this mutation is relatively ancient in the evolution[55].

At the same time, low incidence of heterozygotes for chitotriosidase-mutated allele in African subjects suggests that the persistence of parasitic diseases could have favored the maintenance of the wild-type chitotriosidase gene if chitotriosidase were involved in host defense system against parasites and pathogenic fungi (ref). A support for this hypothesis has recently come from a study showing that humans deficient in chitotriosidase activity are more susceptible to nematodal infection[59]. In their study, in which a total of 216

individuals from South India were genotyped for chitotriosidase, the homozygous condition for the defective allele was associated with the absence of plasma chitotriosidase activity and with an elevated susceptibility to human *Wuchereria bancrofti* filarial infection. But a recent study on Mediterranean and European populations suggest that this mutant allele distribution was determined by random factors instead of selective pressure (e.g. malaria endemicity)[60]. Therefore, the evolutionary origin and maintenance of this chitotriosidase polymorphism may not totally depend on persistence of parasitic disease. Could this suggest that chitotriosidase has other functions affecting its evolution? More studies are needed to address this question.

I.C.1.2. Acidic Mammalian Chitinase

The second human chitinase was purified and cloned by Boot et al. in 2001. Its isoelectric point is around pH 4 to 5, and accordingly it was named as acidic mammalian chitinase (AMCase)[61]. Mouse AMCase has also been clone and characterized, which has a more acid pH optimum, a major pH optimum at 2 and a secondary pH optimum around 3 to 6[62]. Human AMCase has been characterized as an exo-chitinase.

Human AMCase is expressed in the stomach and at a lower level in the lung, whilst mouse AMCase is expressed in tongue, stomach and alveolar macrophages. Similar to chitotriosidase, AMCase encodes a 50-kDa protein containing a 39-kDa N-terminal catalytic domain, a hinge region, and a C-terminal chitin-binding domain. Due to its abundance in stomach, AMCase was assumed to be involved in chitin-containing food digestion. However, in recent years, more studies were done on its function in the lung, especially in allergy associated asthma conditions.

Aligned with chitotriosidase sequences, AMCase has two more cysteine residues implicating formation of a third disulfide bond, which would help inhibiting unfolding under demanding conditions, such as extremely low pH[63].

I.C.1.3. Chitinase Like Protein

Some mammalian proteins in the Family 18 glycoside hydrolases chitolytically inactive, but they share high sequence similarity with chitinases. They lack the catalytic core sequence DXXDXDXE and are unable to finish protonation of the glycosidic bond. However, because the residues that allow for binding of the substrate are conserved, these proteins are able to bind to chitin with considerable affinity and hence are named chilectins or chitinase like protein. One of them, chitinase 3-like 1, is discussed in detail in the following and is involved in many pathological conditions.

Chitinase 3-like 1 (CHI3L1), also known as YKL40 or HC-gp39

Chitinase 3-like protein 1 is a 40 kDa mammalian glycoprotein. The gene for CHI3L1 is located on chromosome 1q31-q32, and consists of 10 exons and spans about 8 Kb of genomic DNA. CHI3L1 is produced by human synovial cells[64], osteosarcoma cells[65], chondrocytes[66], smooth muscle cells[67], macrophages[68], neutrophils[69], colonic epithelial cells[70] and others. Due to the substitution of an essential glutamic acid residue to leucine in the catalytic core, CHI3L1 has no chitinase activity but still can bind to chitin and chito-oligosaccharides with high affinity through a preserved hydrophobic substrate binding cleft. CHI3L1 also interacts with glycosaminoglycans such as heparin and hyaluronan[67]. The putative heparin-binding site is GRRDKQH (residues 143-149)[71]. Furthermore, Bigg *et al.* have recently reported an ability of

CHI3L1 to bind to collagen type 1, 2 and 3[72]. CHI3L1 has therefore been defined as a CLP or chitinase-like lectin (Chi-lectin)[73].

Physiological function

The physiological and biological functions of CHI3L1 are still unclear. However, the studies regarding arthritis, cancer, and liver fibrosis [66, 69, 74] suggest that CHI3L1 plays an important role in the processes of inflammation and tissue remodeling. Although CHI3L1 is not synthesized under state of health, an induction of this protein expression is observed in patients with rheumatoid arthritis and IBD/colorectal carcinoma[75]. This observation suggests that CHI3L1 expression is induced under several inflammatory and malignant conditions [76]. In rheumatoid arthritis patients CHI3L1 plays as an autoantigen, priming CHI3L1 directed T cell to secrete IFN- γ , and depletion of CD⁴⁺ & CD²⁵⁺ Treg cells will significantly increase severity and incidence of the disease [75]. Ling *et al.* [77] showed that stimulation of human articular chondrocytes or skin fibroblasts with CHI3L1 suppresses the cytokine-induced secretion of metalloproteinase (MMP)-1, MMP-3, MMP-13, and the chemokine IL-8. These findings suggest that CHI3L1 may play as a mediator by enhancing the production of proinflammatory cytokines and chemokines, or stimulate acquired immunity system.

On the other hand, similar to bacterial chitin-binding proteins – CBP-21, which enhances chitin accessibility to bacterial chitinases[78], CHI3L1 enhances a potential binding ability of chitinase-producing pathogenic bacteria to chitin. Therefore, it is possible that CHI3L1 may bind to a complex of chitin/CBP that is formed on bacteria and this binding may subsequently enhance the adhesion and invasion of these bacteria to the host body [76]. Mizoguchi's group provided evidence supporting this role by neutralizing

CHI3L1 with anti-CHI3L1 Ab, which significantly suppressed dextran sulfate sodium-induced acute colitis [70].

I.C.2. Fish Chitinases

Chitinolytic activity has long been observed in fish gastric systems. Significant activity has been measured In *Anguilla vulgaris* and *Salmo irrideus* [79]; the elasmobranchs *Squalus acanthias*, *Etmopterus spinax*, and *Raja radiata* and the teleost *Coryphaenoides rupestris* [80]; *Scylliorhinus canicula*, *Raja montagui* [81]; the anchovy *Engraulis capensis* [82]. Gastric chitinases from the Japanese eel, *Anguilla japonica* [83], and the red sea bream, *Pagrus major* [84] were purified and characterized. A constitutive, endogenous, gastric chitinase has been observed in rainbow trout, which appears in the digestive system early in the development and increase proportionally until reaching adult levels at approximately day 50[85-87].

In our lab, Moe et al. was able to purify and characterize a chitinase (Onmy-Chit.01) from rainbow trout stomach[88]. It is a 41KDa O-glycosylated protein. Onmy-Chit.01 is an exochitinase, hydrolyzing N-acetylglucosamine dimers from the non-reducing ends of chitinous substrates. Chitinase activity of Onmy-Chit.01 is dependent on temperature and pH (optimal condition 25°C, pH4.5) and inhibited by allosamidin.

It was believed that the observed chitinolytic activity from the vertebrate gastric system was due to lysozyme. However, when we purified both lysozyme and chitinase from rainbow trout stomach the data clearly showed that greater than 90% of the activity was due to Onmy-Chit.01. Thus I conclude that the chitinolytic activity observed in rainbow trout stomach is due to an authentic chitinase.

It is not surprising that rainbow trout has such high levels of chitinase activity, since it is insectivorous throughout their life, relying on chitinous invertebrates for most of their diet [88]. However, studies have shown that the apparent digestibility of dietary chitin by rainbow trout is extraordinarily low and the addition of chitin to the diet suppresses growth[85, 87]. This anomaly between high activity and low digestibility could be due to the nonnative chitin substrate used in experiments but further experimentation is needed.

I.D. Chitinase's Function

Chitinases exist in all phyla of organisms, from bacteria, virus, plants, invertebrate and vertebrate, but many of these organisms do not contain chitin. Various biological functions have been attributed to the chitinases in the various species. Invertebrate chitinases are involved in a morphogenic processes of their chitinous coatings, arthropods molting from their exoskeleton, nematodes hatching from their chitin-coated eggs [63]. However, in vertebrates, based on current literatures and the fact that vertebrates usually do not contain chitin as their structural elements, two functions have been proposed for vertebrate chitinases - as a food processing enzyme and as an immune defense factor.

I.D.1. Food processing

Chitin is the second most abundant carbohydrate in the world, it is an important structural element for many organisms, and it is also a good nutrient resource for many organisms, especially for many marine bacteria, that can grow on chitin as a sole carbon and nitrogen source[89]. Carnivorous plants also contain chitinase in their digestive juice, such as in *Nepenthes alata*, where chitinase in its pitcher fluid helps the plant to supplement its nitrogen intake[90].

However, whether vertebrates use chitin as a nutrient resource is not conclusive. Human consumption of adult insects and larvae is very popular in most tropical and some temperate countries. Nevertheless, study on how human digest chitin-containing food is very limited. Multiple studies showed that AMCase protein is expressed in both human and mouse stomach mucosa but at levels much lower than observed in insectivorous mammals[91, 92]. Furthermore, a study showed that AMCase exerts activity in gastric juice, the possibilities were excluded that activity is from gastric flora; but 20% of subjects in this study did not have chitinase activity in their gastric juice, which could be due to the fact they do not consume chitin-containing food[93].

As mentioned in the previous section, in rainbow trout, there is a contradiction between high gastric chitinolytic activity and low chitin food digestibility, which could presume that chitinases in vertebrate gastric system may have other functions besides processing of chitinous food.

In fact, chitin performs as a poor nutrient resource for fish. Lindsay's survey of gastric chitinase activity in fish [87] showed no correlation between a chitin-eating feeding habit and gastric chitinase activity. He did find a minor correlation between the mode of feeding and gastric chitinase activity: those fish that possess a means of disrupting the prey mechanically (such as a modified buccal cavity or pharyngeal teeth) did not show chitinase activity. He theorizes that the enzyme is a food-processing enzyme. This function would be especially important in larval fish, a lifestage during which most fish species rely on chitinous prey (regardless of adult diet) and, because of the small size of the animal, are particularly vulnerable to gut blockages. Even

superficial breakdown of the chitin in the prey items could facilitate movement of the prey through the gut, protecting against potentially fatal gut blockages.

However, in some studies, fish growth can be enhanced by the addition of chitin in feed, which means chitin is one of their nutrition resource [94]. Red sea bream, Japanese eel and yellowtail were fed diets supplemented with either chitin, chitosan or cellulose. The chitin supplemented diet yielded a significantly higher growth rate than the control diet. The cellulose diet was not different from the control and the chitosan diet appeared to inhibit growth.

One possibility is chitin was digested and absorbed by intestinal bacteria. Multiple studies showed chitinolytic bacteria exist widely in fish intestine, most of them belong to *Vibrionaceae* family and perform as endochitinases [95, 96]. They may also act as anti-fungal agents since the food is retained for long periods in the stomach of fish.

I.D.2. Immunity

Due to the fact that many vertebrate pathogens use chitin as structural elements, it is not hard to associate chitinase expression with immune defense. As a matter of fact, the role of chitinase as a fundamental player in the immunity defense of plants has been long recognized.

I.D.2.1. Defense Mechanism in plants

Chitinases are an important component of plant pathogenesis-related proteins (PRs). PRs are plant defense proteins that are induced after pathogenic infection (viruses, bacteria, fungi and pest) or environment stress (drought, salinity, wounding, heavy metals et al.). They provide the host plant with enhanced capability to limit subsequent

infections by inhibiting pathogen growth, multiplication and spread. Their toxicity is due to proteinase-inhibition and membrane-permeability[97].

In the face of wide geographic variation in pathogens, plants are likely to encounter novel resistance or inhibition mechanisms that place plant chitinases under positive selection to enhance their capability to evolve to different substrate specificities[98]. Studies have found a greater rate of amino acid replacement in the active site and substrate binding cleft of class I (plant) chitinase, compared with the structural and functional relatives class III chitinase[98]. The disproportionately high abundance of amino acid replacement in the active site could suggest that fungi or insect directly defend against chitinase through enzymatic or other forms of chemical resistance, which forced plant chitinase to evolve and generate new structures to defend the host.

One mechanism for plant chitinases to work as a defense molecule is to hydrolyze the chitin component in pathogen cell wall, thereby inhibit intrusion and growth of pathogen. Another way is for breakdown products of chitinolytic reaction to act as an “elicitor: for further immune defense[97]. Chitin-oligomers may induce classic pathogen-associated molecular patterns (PAMP) responses such as the activation of MAPK cascades and alterations in protein phosphorylation or gene transcription, even without the presence of pathogens[99-101].

I.D.2.2. Mammalian Chitinase in Innate Immunity Defense

Chitin-containing pathogens of vertebrates include primarily fungi and nematodes. In order to defend against these pathogens, vertebrates need an efficient first line defense - the innate immunity system. Phagocytosis is one of the most essential ways that the immune system clears up invading pathogens. When a macrophage ingests a pathogen,

the pathogen becomes trapped in phagosome, which then fuses with a lysosome. Within the lysosome, enzymes and toxic peroxides digest the invader.

By definition, lysosomes are membrane-bound cytoplasmic organelles involved in intracellular protein degradation. They contain an assortment of soluble acid-dependent hydrolases and a set of highly glycosylated integral membrane proteins[102]. Lysosomes are morphologically heterogeneous, they are currently distinguished from other organelles on the basis of an operational definition, which describes them as membrane-bound acidic organelles that contain mature acid-dependent hydrolases and lysosomal-associated membrane proteins (LAMPs) but lack mannose 6-phosphate receptors (MPRs)[103]. Most or all of these characteristics are shared with a group of cell type-specific organelles that includes lytic granules, platelet-dense granules, basophil granules, and neutrophil azurophil granules etc. These shared traits suggest that these specialized organelles may be biogenetically related to lysosomes[102].

The first identified vertebrate chitinase-human chitotriosidase was found in a lysosome-disorder disease (Gaucher's disease). Human chitotriosidase seems to be expressed exclusively by human phagocytes, such as macrophages and neutrophils. In human neutrophils, chitotriosidase is present in specific granules. There are primarily four kinds of granules in neutrophils, azurophil (also known as primary) granules, specific (also known as secondary) granules, gelatinase (also known as tertiary) granule and secretory granules. These classes of granule are formed sequentially during different stages of neutrophil differentiation in the bone marrow. The granules are classified according to their protein content and their differential ability to be exocytosed after neutrophil activation by inflammatory stimuli or phagocytosis of invading

microorganisms. Along with chitotriosidase, there are lactoferrin, cathelicidin, lysozyme etc. in specific granules[104]. *In vitro* stimulation of neutrophils with granulocyte macrophage-colony stimulate factor (GM-CSF) results in simultaneous release of chitotriosidase and lactoferrin[105].

Monocytes do not express chitotriosidase. Chitotriosidase mRNA is expressed only at later stages of differentiation of monocytes to activated macrophages in culture [51]. However, mouse chitotriosidase has a very different tissue distribution when compared to humans. It seems to be absent in phagocytes, but expressed predominantly in the gastrointestinal tract (stomach and tongue), brain, skin, bone marrow, testis, and kidney[106]. As mentioned before, mouse AMCase is upregulated after alveolar macrophages are stimulated along with Th2 cells activation.

Human chitotriosidase is not a house-keeping enzyme[42], it is released under pathological conditions and inflammatory immune response. Cytokines IFN- γ and TNF- α up-regulated chitotriosidase gene expression in human macrophages, whereas IL-10 led to remarkable suppression of chitotriosidase expression[107].

Classic innate immunity receptor Toll Like Receptor (TLR) has been studied with human chitotriosidase expression. For TLR-2 triggering, peptidoglycan, lipoteichoic acid, zymosan, and the synthetic ligands macrophage-activating lipopeptide-2 (MALP-2) and Pam3CSK4 have been used. For TLR-3, poly(I:C) has been used. In both circumstances, activation of TLRs on monocytes prevented induction of chitotriosidase. Stimulation of TLR-4 and TLR-9 was accomplished with lipopolysaccharide (LPS) from *Salmonella minnesota* and CpG, respectively. LPS was observed to upregulate chitotriosidase expression approximately 300 fold after 4 hours compared to untreated cells[107], but re-

stimulation with LPS does not induce further expression of the enzyme[63]. This could imply that further upregulation of chitotriosidase in human macrophage needs breakdown products of chitin, which is the case for AMCase in alveolar macrophage.

More direct evidences of chitinase on anti-fungi activity are from studies involving *in vitro* fungal infection. Increased chitinolytic activity has been reported in tissues and plasma of guinea pigs infected by *Aspergillus fumigatus* [108]. As an analog in human neonates, increased chitinase activity was observed in plasma and urine with fungal and bacterial infection. Improvement in their clinical condition was associated with a decline in chitinase activity[109, 110]. When incubated with fungi, it can inhibit growth of *Cryptococcus neoformans*, cause hyphal tip lysis in *Mucor rouxii*, and prevent the occurrence of hyphal switch in *Candida albicans*[105]. Furthermore, recombinant human chitotriosidase can improve survival rate in neutropenic mouse models of systemic candidiasis and aspergillosis[105].

I.D.2.3. Mammalian chitinase in allergic responses: Asthma

Asthma is a highly complicated disease that is still poorly understood. Allergic asthma is thought to result from maladaptive inflammatory responses to ubiquitous environmental proteins in genetically susceptible persons. One asthma theory suggests that the disease is caused by the body responding to a parasitic threat that is not in fact there. More specifically, allergic asthma is a chronic inflammatory disorder of the airways mediated by CD⁴⁺ T cells polarized to a type 2 helper (Th2) phenotype. Th2 cytokines drive the cardinal features of the disease: pulmonary eosinophilia, elevated concentrations of serum IgE, airway hyperresponsiveness, and excessive production of mucus in the airways. In particular, the Th2 effector cytokine interleukin-13 has a critical

role in mediating airway hyper-responsiveness and mucous metaplasia, the elements of allergic asthma that are most closely linked to disease expression. These pathologic immune responses have long been recognized to mirror beneficial immune responses to helminth infection[111].

Despite the important roles chitotriosidase plays in all sorts of diseases as mentioned before, it has been pointed out that chitotriosidase has no association with bronchial asthma [112]. However, AMCase has been shown to be expressed in exaggerated quantities in both a mouse model of asthma and human asthma working as a mediator on a T helper-2 (Th2)–specific, interleukin-13 (IL-13)-dependent pathway[113]. Neutralization of the chitinase, with either antibodies or the chitinase inhibitor allosamidin, ameliorated airway inflammation as well as airway hyperresponsiveness. This could implicate that chitinase can be used as a therapeutic target in allergic asthma.

Another study demonstrates that chitin particles induce the tissue infiltration of eosinophils and basophils when given to mice; tissue macrophages serve as sensors to chitin. Accumulation of these cells was not affected by absence of TLR (Toll-like-receptor) mediated lipopolysaccharide recognition. However, if chitin was pretreated with AMCase or mice were overexpressing AMCase, tissue filtration would not occur[114]. This means AMCase breaks down chitin to downregulate chitin-induced allergic innate immune responses, thus removing the stimulus for further eosinophil and basophil recruitment. Eosinophils and basophils are IL-4&IL-13 expressing cells, which can induce AMCase expression.

Thus we could presume a model of chitin induced inflammatory reaction. Chitin particles were introduced to host's lung, stimulating local macrophage to express

AMCase and attract the tissue penetration of eosinophils and basophils, those cells release IL-4&IL-13, which stimulate macrophages to express more AMCase. AMCase breaks down chitin and down-regulate this priming process. But under pathological allergic conditions, there is no real chitin-containing pathogen available, this down-regulation cannot be processed, overloading AMCase along with other Th2 cytokines are released to the lung, which cause asthma.

On the other hand, some studies suggest quite opposite results on inflammatory responses upon chitin stimulation, it boosts the Th1 response and downregulates the Th2 response [115].

The contrary results of these studies could be due to the differences in mouse models, chitin preparation and delivery, and experimental artifacts. It is also important to note that chitin in nature is rarely present by itself; rather it always associates with β -glucan and/or proteins. Inflammatory responses generated under experimental conditions may not represent *in-situ* conditions.

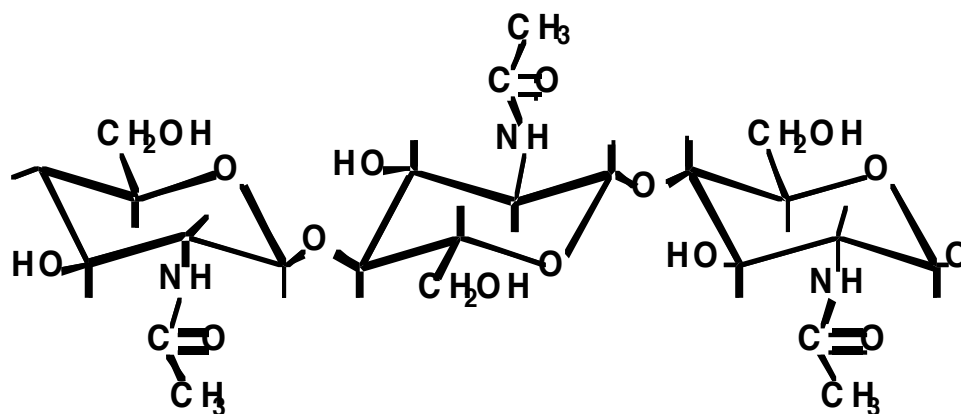


Figure I-1. Chitin molecular structure: Poly-(β (1,4)- N-Acetylglucosamine).

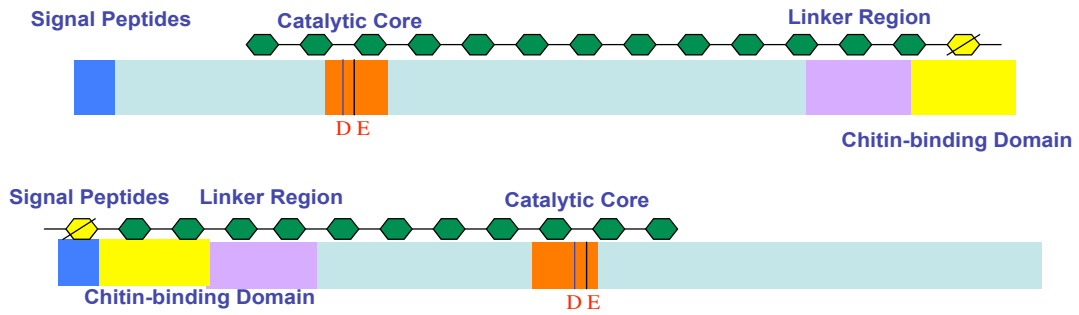


Figure I-2.Family 18 Chitinase Domain Structure. A. vertebrate chitinase; B. Bacterial chitinase; Dark green hexagon represents NAG residue, Yellow hexagon represents reducing end.

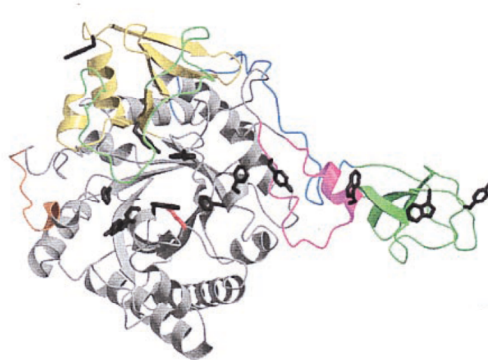


Figure I-3.Structure of Family 18 chitinase-*Serratia marcescens* ChiB. TIM barrel (gray),the linker (blue), and the CBD (green). [28]

I.E. Objectives

Previous studies have shown that rainbow trout produce a 41KDa glycosylated protein that operates as an exochitinase in stomach. It hydrolyzes N-acetylglucosamine dimers from the non-reducing ends of chitinous substrates. The optimal enzymatic condition of this gastric chitinase is 25°C and pH 4.5. However, rainbow trout kidney exerts chitinolytic activity at pH 7.5. Moreover, my investigation of chitinase in rainbow trout will not only focus on gastric tissues. Instead, I will examine the chitinase expression in various organs and investigate its function other than being a digestive enzyme.

Given the preceding background, I propose the following hypotheses:

Hypothesis 1: There are at least two chitinase genes in the rainbow trout genome. Each gene encodes a chitinase that may perform at different environmental conditions.

It was necessary to clone and characterize the cDNAs that code for the enzymes responsible for the observed chitinase activity. Investigation of their tissue and cellular origins provide hints at their functions.

Hypothesis 2: The gene structure of vertebrate chitinases is similar, at least in the protein coding region.

Chitinase possesses some critical amino acid sequences for their enzymatic activity, any mutation happened in the catalytic core will cause the protein to lose its chitinolytic activity. This conserved region could mean that there is less mutation on gene coding region, namely, conserved genomic structure. In order to test this hypothesis, it is

necessary to sequence rainbow trout chitinase genes. An established BAC library will be used for the greater part of these studies.

Hypothesis 3: A conservative regulatory region (promoter) may exist throughout the vertebrates.

Chitinase can be observed in the gastric portion of animals throughout the vertebrate classes. The regulatory mechanism of this enzyme could be similar too. Existing genomic information of model vertebrate animals can be used to compare with rainbow trout chitinase genes. Transcription factor binding sites and evolutionary conserved regions (ECR) investigation will provide information on control and regulation of chitinases in vertebrates.

Hypothesis 4: Chitinase has multiple functions in vertebrates. Perhaps involved in food processing of chitin containing diets, its primordial function maybe as a factor of the innate immune system.

In order to disclose the mystery of chitinase function in innate immunity, it is important to know whether chitinases will change their expression patterns under certain stimulation conditions. Both *in vivo* and *in vitro* stimulation studies using known immunogens will be examined for this purpose.

Chapter II Cloning and Expression of Rainbow Trout Chitinases

II.A. Introduction

Chitinolytic activity (i.e. the ability to cleave the $\beta(1-4)$ linkage in poly N-acetylglucosamine) has been documented in all kingdoms of life; the earliest record can be traced back to 1929 in bacteria[116]. In vertebrates, chitinolytic activity had been observed in the gastric system for many years[117], however, the first vertebrate chitinase gene was cloned only a dozen years ago and named human chitotriosidase[51]. Seven years later, in collaboration with our laboratory the second human chitinase gene was cloned—acidic mammalian chitinase (AMCase)[61]. These two chitinase genes have very different tissue distribution patterns and are involved in distinct pathological processes[113, 114]. Furthermore, their counterparts in mice have also been cloned and show additional tissue distribution expression patterns [61, 106].

In both humans and mice, AMCase is expressed in alveolar macrophages and the gastrointestinal tract. But for mouse and human chitotriosidase, which share 78% identity in their amino acid sequences[106], the tissue distribution expression patterns are very different. Human chitotriosidase is expressed exclusively by professional phagocytes, whereas mouse chitotriosidase is expressed in the gastrointestinal tract, the tongue, forestomach, brain and Paneth cells of the small intestine[92, 106].

Multiple chitinase genes have also been found in other vertebrates. Two distinct chitinase cDNAs were cloned from toad stomach and pancreas [118, 119] and three chitinase cDNAs were cloned from the Japanese flounder[120]. Based on BLAST

analyses, multiple chitinase genes are found in all other vertebrates, including three in zebrafish, two in frog, and three in chicken.

Based on this data I assert that most vertebrates contain multiple chitinase genes. One is primarily expressed in gastric system and another is expressed in cells from the immune system. The biological significance of these chitinase genes is largely unknown. The gastric chitinase may contribute to food processing, but may also be involved in protection against fungal pathogens. Expression of chitinases as members of the innate immune system may participate in defense against chitin-containing pathogens, and work as part of the first line defense [59, 105, 121].

Recent studies on chitinases from vertebrates have focused mainly on mammalian systems. Both chitotriosidase and AMCase are involved in pathological conditions and their expression patterns are useful for diagnosing various diseases. Expression of these two chitinases does not overlap and may reflect different biological targets. AMCase was expressed extensively in lung of asthma patients or the alveolar macrophages in a mouse asthma model [106, 114], while chitotriosidase was not detected in the lung of these conditions [106, 114]. Similarly, chitotriosidase expression level was a thousand fold higher than usual in plasma of Gaucher's disease patient, whilst AMCase was not be detected in the plasma of the same patient [63].

These facts raise a very important question, why do organisms bother to make two enzymatically similar proteins in different conditions? Furthermore, what is the relationship of these two enzymes? Do they complement each other functionally? Do they derive from the same origin? Are they enzymatically identical? To address these questions, we investigated the presence and expression of chitinases in finfish. The

finfish is considered as the lower vertebrate, it was believed that large scale of gene duplication happened at this evolutionary step.

Chitotriosidase has not been well studied in non-mammalian animals, though one of three Japanese flounder chitinase cDNAs cloned by Kurokawa et al. was predicted to be phylogenetically close to chitotriosidase, but they did not localize its expression [120].

We chose rainbow trout as our study model, because it has highest gastric chitinase activity in all the vertebrate species we have examined[61]. Rainbow trout has long been documented to possess a constitutive, endogenous, gastric chitinolytic activity[85, 122]; Because of their insectivorous behavior, rainbow trout are known to encounter high amounts of dietary chitin. Our lab successfully purified chitinase protein from adult rainbow trout stomach, characterized its enzymatic activity and developed a polyclonal antibody designated as Anti_Onmy-Chit.01, which recognizes a shared epitope in all vertebrate chitinases [61, 123, 124].

The current study set out to obtain full-length rainbow trout chitinase cDNAs and study its tissue and cellular expression pattern. We found two rainbow trout chitinase cDNAs, which shared 56% similarities in their amino acid sequences. One is expressed in gastric gland of stomach and the other is primarily expressed by the myeloid cell lineage of immune systems. The evolutionary relationship of the two chitinases with other known vertebrate chitinase are examined and the functional significance of their expression patterns is discussed.

II.B. Material & Methods

II.B.1. Experimental organism

Rainbow trout (*Oncorhynchus mykiss*) fingerlings were obtained from Clear Spring Foods Inc. (Buhl, ID) and grown to average weight around 500g prior to sacrifice. They were held in our Aquaculture Research Center and exposed to simulated natural photoperiod conditions at temperatures 15°C, which mimics the conditions fish experience in the wild.

II.B.2. Production of Tritiated Chitin and Radiometric Assay for Chitinase Activity

Acetyl-[³H]-chitin was prepared by reacylation of chitosan with tritiated acetic anhydride as described by [125] and modified by [126]. Crabshell chitosan was dissolved in 10% glacial acetic acid to a final concentration of 1% (w/v) and then methanol (10 times the volume of the chitosan/acetic acid solution) was added. Acetic anhydride (10 mCi per gram of crabshell chitosan in excess unlabeled acetic anhydride) was added to the stirring methanol solution and the solution was allowed to gel. The newly formed gelatinous solid was then homogenized in methanol with an Ika Ultraturrax T-50 homogenizer. This suspension was washed with methanol until the fluid showed no radioactivity above background. The chitin suspension was stored in 0.01% sodium azide at 4°C at a concentration of approximately 15 mg/mL. The specific activity of the reacylated chitin was determined by the consecutive degradation of the substrate into GlcNAc monomer by chitinase (0.215 units/mL from *Serratia marcescens*) in 0.1 M NaPO₄, pH 6.0 and β-glucuronidase (6592 units/mL from *Helix pomatia*) in 0.1 M

NaPO₄ pH 6.0. Total GlcNAc was determined based on a standard curve of GlcNAc by the method of microtiter colorimetric assay[126].

Chitinase activity was assayed using the synthesized tritiated chitin. The 100 μ L assay solution consisted of 5 μ L of 1 M ammonium acetate pH 4.5, 15 μ L chitin suspension, assay sample and water. The assays were incubated in 1.5 mL microfuge tubes in an Eppendorf thermomixer at 1,400 cycles•minute⁻¹. The constant action of the thermomixer kept the chitin constantly suspended and therefore available for interaction with the assay sample. The enzymatic reaction was stopped and insoluble chitin was precipitated with 300 μ L of 10% TCA(trichloroacetic acid). This mixture was centrifuged for 5 minutes at 14,000 g and 200 μ L of the supernatant was carefully removed so as not to disturb the chitin pellet and the tritium of that solution was measured in β counter (Beckman LS 1801). The basic unit of this assay is μ g GlcNAc released hour⁻¹.

II.B.3. Tissue Distribution of Chitinase Activity

A survey of rainbow trout tissues was conducted to determine the distribution of chitinase activity. The gastro-intestinal tracts of five rainbow trout yearlings were divided into seven sections (four gastric, three intestinal; arbitrary sectioning anterior to posterior). The gastric mucosa of one of the fish was scraped from the gastric tissues and processed independently from the gastric tissues. Other tissues (liver, kidney, head kidney, pancreas, heart, ovary, brain, gills, muscle, and gall bladder) and two fluids (blood serum and bile) were also obtained from one of the five adult rainbow trout. All samples (except bile and blood) were extracted immediately in 0.5% acetic acid, centrifuged at 14,000 g, and filtered through miracloth. Bile and blood were assayed directly. Supernatants were assayed for chitinase activity and total protein.

II.B.4. Protein Assay

Total protein was determined with a BCA kit (Pierce Chemical) according to the manufacturer's instructions using bovine serum albumin (0-20 µg) as the standard.

II.B.5. RNA preparation

Tissues were collected from rainbow trout that have been anesthetized with MS222 (3-aminobenzoic acid ethyl ester, 150mg/l water), dissected and frozen on dry ice. Each tissue was ground into a powder in a liquid nitrogen chilled mortar. 50-100 mg tissue powders were mixed with Trizol (Invitrogen) in Lysing Matrix D tube (Bio101 systems) and ground in FP 120 FastPrep cell disruptor (Bio101 systems). Total RNA was obtained by following Trizol manufacturer's instructions and quantified by using UV spectrophotometry (Beckman DU-690).

II.B.6. Amplification of the full-length Gastric Onmy-Chit.01 chitinase

The cDNA library was synthesized using 2 µg total RNA from specific tissues with Poly dT+ primers using SuperScript II reverse transcriptase (Invitrogen, CA, USA) according to the manufacturer's instructions. An aliquot of 100 ng of the first-strand from each cDNA was amplified with gene specific degenerate primers. Three pairs of cDNA degenerate primers (suggest by R. Boot, personal communication) were used.

Table II-1. Degenerate primers used for Onmy-Chit.01 amplification.

	Name	Sequence	Start	End	Length
1	CYFT-sense:	5'-TGYTAYTWYRCCARYTGGKC-3'	90	110	21
2	GGW-sense:	5'-AYHCTSYTRKCYRTYGGAGG-3'	287	307	21

3	GGW-antisense:	5'-AYRGM YARSAGIRTYTTYAG-3'	301	282	20
4	FDG-sense:	5'-AYRRSTTYGAYGGVCTBRA-3'	403	421	19
5	FDG-antisense:	5'-TYVAGBCCRTCRAASYRT-3'	421	402	20
6	MTYD-antisense:	5'-CCRTGIARRTCRWAIGHCA-3'	661	643	19

Degenerate Code: **R**= A / G, **Y**= C / T, **M**= A / C, **K**= G / T, **S**= C / G, **W**= A / T, **B**= C / G / T, **D**= A / G / T, **H**= A / C / T, **V**= A / C / G, **N**= A / C / G / T

PCR reactions with degenerate primers and the stomach cDNA library as a template were set up and PCR products were separated on 1% agarose gel, purified by QIAquick Gel Extraction Kit (Qiagen Inc., CA, USA), cloned into pCRII-TOPO vector (Invitrogen) and sequenced on an ABI 377 DNA sequencer (Applied Biosystems). To obtain the full length chitinase cDNA, rainbow trout stomach 5'- and 3'- RACE (rapid amplification of cDNA ends) cDNA libraries were constructed using the SMART RACE cDNA amplification kit (BD, Biosciences, CA, USA) following the manufacturer's instructions using 2µg total RNA. Gene-specific primers (Table 2) for 5'- and 3'- RACE were designed based on the 500 bp sequence obtained from degenerate primer amplification, and were used in conjunction with the universal primer mix (UPM) primer (adaptor primer from kit. PCR products were sequenced as described before and the full-length cDNA assembled using MacVector (Accelrys Inc., CA, USA). This cDNA was designated as Onmy-Chit.01.

Table II-2. Primers used for cDNA RACE.

	Name	Sequence	Start	End	Length
1	Chit.01 5'RACE	5'-CACTGGACACCATTGCTGTGAACGG-3'	351	327	25
2	Chit.01 3'RACE	5'- CGAATGGGACGATGAGAACTCTACGG-3'	221	247	27
3	Chit.02 5'RACE	5'-CACCGTTGGCTGACGCTCCGAC-3'	907	886	22

4	Chit.02 3'RACE	5'-GCTGGGAAAGGGACCATTGATTCTGG-3'	604	629	26
5	Pepsinogen 5'RACE	5'-CTGAATGGAAACGGGCTTGTTGG-3'	428	406	23
6	Pepsinogen 3'RACE	5'-CTACTGCTCCAGCCAGG-3'	335	351	17

II.B.7. Head Kidney cDNA library screening and full-length characterization of Onmy-Chit.02 chitinase

Onmy-Chit.01 cDNA sequence was blasted against an LPS stimulated macrophage cDNA library [19]. Five sequences homologous to Onmy-Chit.01 cDNA were found. These five sequences were assembled into one Contig using MacVector (Accelrys Inc., CA, USA), providing a 1000 bp cDNA. As described in Onmy-Chit.01 cloning, rainbow trout head-kidney 5' RACE and 3'RACE libraries were made and gene specific primers were designed based on 1000 bp consensus sequence, cloning and sequencing were performed as described before. The primers are listed in Table 2. The full-length cDNA from the head kidney was designated Onmy-Chit.02.

II.B.8. Rainbow trout Pepsinogen amplification

In order to obtain a rainbow trout transcript that exhibits gastric expression to serve as a positive control, the cDNA for pepsinogen was cloned. Degenerate primers were designed based on alignment of other species (human, pig, chicken, frog, winter flounder, brook trout)'s pepsinogen cDNAs, forward primer: 5'-(AG)A(CT)(CT)TITGG(GA)TICCI(AT)(CG)I(GA)T(AGTC)TA-3'; reverse primer: 5'-A(CT)I(GT)(GT)IA(GCA)IGT(AG)TC(GA)TAIGC)CIA-3'. A 180 bp amplicon was

determined for this gene specific product. As described above, 5' and 3' was used with specific primers designed within this 180 bp sequence to clone the full sequence of rainbow trout pepsinogen cDNA. The primers are listed in table 2. RACE PCR bands were purified and cloned as described before. This cDNA was designated Onmy-Chit.02.

II.B.9. Chitinase mRNA Tissue Distribution

Chitinase expression levels were determined at the transcript levels using real-time fluorescence-based quantitative RT-PCR assays (qPCR). Total RNA isolated from each tissue or plasma using a modified acid-phenol extraction method (TRI REAGENT, MRC, Inc.) was reverse-transcribed using random hexamers and MMLV reverse transcriptase. qPCR was performed using TaqMan fast universal PCR master mix (Applied Biosystems) and gene-specific primers and probes. Amplification reactions were carried out using an ABI 7500 Fast Sequence Detection System (PE Applied Biosystems). For each reaction an amplification plot of fluorescence signal versus cycle number was generated and the C_T (the fractional cycle number at which fluorescence passes a baseline threshold value) was calculated. The expression levels of unknown samples were determined by comparing C_T values normalized to the amount of 18s rRNA in each sample. qPCR probes for Chit01& Chit02 (Figure II- 2) were not shown to detect each other's plasmid when plasmid concentration is lower than 0.1ng/ul in preliminary testing experiment.

Table II-3.qPCR primers and probes sequences for Onmy-Chit.01 & Chit.02

	Name	Sequence	Start	End	Length
1	Chit01qPCR forward	5'-TTGGCACTCAGCCGTTAC-3'	316	334	19

2	Chit01qPCR reverse	5'-TGATAACGCTGCTGATGAACGT-3'	387	366	22
3	Chit01qPCR Probe	5'-TGTCCAGTGCAGCCAACCGCC-3'	343	363	21
4	Chit02qPCR forward	5'-TCTATAACTGTGCTAACGGCATCAC-3'	1349	1373	25
5	Chit02qPCR reverse	5'-CATTTGCAGCTGTCACTGAAGAC-3'	1424	1402	23
6	Chit02qPCR Probe	5'-TGGATCCAGAGCTGCCCCGC-3'	1375	1394	20

II.B.10. Northern Blotting

Total RNA (20 µg) from all samples were enriched for mRNA by oligo(dT) Cellulose (New England Biolabs Inc.). Briefly, oligo(dT) Cellulose powder (200 mg) was hydrated in loading buffer and then incubated with total RNA for 60 min at room temperature. Pellets were washed twice in loading buffer and then eluted by elution buffer. mRNA enriched RNA was precipitated by 100% ethanol, washed with 75% ethanol, and resuspended in DEPC-H₂O. mRNA enriched RNA was loaded on formaldehyde-agarose gel. Briefly, 100 ml of a 1% agarose / 2.2M formaldehyde gel solution was prepared as follows: 1.2g of agarose was added to 72ml DEPC-H₂O and dissolved by boiling. The solution was allowed to cool to 55°C and 10ml of 10X MOPS buffer and 18ml of 37% formaldehyde were added to the agarose solution. 5µg of these mRNA enriched total RNA were mixed with 10X MOPS loading buffer and loaded on formaldehyde-agarose gel. RNA was fractionated by electrophoresis at 5V/cm for 10hrs. 5µg of a 0.5-9Kb RNA

ladder (New England Biolabs Inc.) was used as a size marker. After electrophoresis, the gel was stained in 0.5 µg/ml ethidium bromide for 30 min and scanned using a Typhoon Imager to assess the integrity of the RNA and to confirm that equal quantities of RNA were loaded by UV visualization of the ribosomal bands.

After electrophoresis, the gel was incubated for 15 min in 20X SSC and then RNA was blotted to positively charged nylon membranes (Schleicher & Schuell Inc., NH, USA) by capillary transfer with 20X SSC overnight. RNAs were cross-linked to membranes by UV irradiation using a stratalinker at optimal condition. The position of RNA ladder lane was marked and cut out from membrane and stained with methylene blue for 5 mins.

Probe generation is described below.

Table II-4. Primer sequences used for generating northern blotting probes.

	Name	Sequence	Start	End	Length
1	Chit01 antisense probe forward	5'- GAATTCTAATACGACTCACTATAGGGCAGACGAG GAAGGTTGGTGTTC-3'	548	527	22
2	Chit01 antisense probe reverse	5'-TCAGCCGTTACAGCAATGG-3'	323	342	20
3	Chit01 sense probe forward	5'- GAATTCTAATACGACTCACTATAGGGTCAGCCGT TCACAGCAATGG-3'	323	342	20

4	Chit01 sense probe reverse	5'-CAGACGAGGAAGGTTGGTGTTC-3'	548	527	22
5	Chit02 antisense probe forward	5'- GAATTCTAATACGACTCACTATAGGGACGAGCCG AGGTCATCATTTTC-3'	1477	1497	21
6	Chit02 antisense probe reverse	5'-GGGGAATCTGCTTTGACATACAGC-3'	1317	1340	24
7	Chit02 sense probe forward	5'- GAATTCTAATACGACTCACTATAGGGGCTGTATG TCAAAGCAGATTCCCC-3'	1317	1340	24
8	Chit02 sense probe reverse	5'-ACGAGCCGAGGTCATCATTTTC-3'	1477	1497	21

For RNA probe generation, PCR reactions were set-up with chitinase specific primers (Table 4) and stomach cDNA library as template for Onmy-Chit.01, and head-kidney cDNA library as template for Onmy-Chit.02. PCR products were separated on an agarose gel, purified by QIAquick Gel Extraction Kit (Qiagen Inc., CA, USA). To generate the antisense and sense hybridization probes labeled with DIG, transcription reactions were set up as follow: T7 polymerase (Ambion) 1µl, 5X transcription buffer 4 µl, 100mM DTT 2µl, DIG-NTP 2µl, RNase inhibitor (Invitrogen) 1µl, DEPC-H₂O 9µl, purified product

from above 100ng/μl 1μl. The reaction was incubated for 2 hrs at 37°C and purified by using chroma spin column (Clontech, CA).

The membrane for blotting was prehybridized with 10ml DIG Easy Hyb (Roche) at 68°C for 30min, then hybridized with 3.5ml fresh DIG Easy Hyb containing 350ng probe for 16hrs. After incubation, the membrane was washed twice in low stringency buffer (2X SSC containing 0.1%SDS) for 10 min at room temperature, and twice with high stringency buffer (0.1X SSC containing 0.1%SDS) at 68°C for 30min. As a final wash, membrane was gently rocked for 2 min at room temperature with washing buffer (0.1M maleic acid, 0.15M NaCl, 0.3% tween 20, pH7.5). Blocking was performed using blocking solution (Roche) for 30 min, afterward, with 20μl new blocking solution containing 2μl anti-DIG-AP (Roche) antibody for 30 min. After blocking, the membrane was washed twice (2 X 15min) with washing buffer, equilibrated in 20 ml detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5) for 3min. CDP-star (Roche) was applied in drop-wise manner onto the membrane which was placed between two acetate sheets until the entire surface of membrane was covered, incubating for 5 minutes. The membrane was exposed to X-ray film in dark room at room temperature for up to 10 mins and processed in a Kodak film processor.

II.B.11. Polyclonal Antibody Production

Antibodies were raised (Bioworld, Inc., Dublin, Ohio, USA) against purified Onmy-Chit.01[88]. Dry lyophilized protein (500 μg) was reconstituted in saline and mixed with 0.5 ml of Freund's complete adjuvant. The mixture was inoculated at multiple sites subcutaneously in the inguinal and axillary regions and intramuscularly in the hind limbs

of the rabbit. Three subsequent injections (at 3 weeks, 5 weeks and 7 weeks) used 250 µg of Onmy-Chit.01 were injected as previously described.

The rabbit serum that was collected after the injection cycles was compared with pre-immune serum from the same rabbit. Western blots of 12% SDS-PAGE of pure protein were sectioned and probed with both pre (1/10,000 dilution) and post-immune serum (1/500,000 dilution). The post-immune serum was also serially diluted and the dilutions used to probe pure protein (5 µg in each lane) on western blot as above in order to determine the optimal dilution.

Anti-progastricsin antibody was generated as describe before[127].

II.B.12. N-terminal Amino Acid Sequencing

Lyophilized, salt-free Onmy-Chit.01 protein (4 mg) was dissolved in 1 mL of 10 mM Tris-HCl, pH 8.0 containing 10 mM DTT, 1 mM EDTA, and 8 M urea. The sample was incubated at 37°C for 30 minutes. Iodoacetamide (1.54 mg) containing 50 µCi [^{14}C] iodoacetamide (final specific activity 0.77 µCi/µmole) in ethanol was added to the protein solution. After incubation in the dark for one hour at 37°C, the sample was diluted 10 fold with 10 mM ammonium acetate and dialyzed (3 x 1 liter changes until no further radioactivity above background was detected) overnight at 4°C in the dark. The sample was lyophilized for 24 to 48 hours. Edman degradation on 100 µg of this material was performed on a Beckman LF3000 gas-phase protein/peptide sequencer coupled to a Beckman System Gold phenylthiohydantion analyzer. Proteins and peptides were immobilized to the appropriate glass fiber support prior to analysis. Peptide immobilized on PVDF membranes were placed between Zytex netting on the sample cartridge for the sequencing.

II.B.13. Western-Blotting

Protein separation was performed using BioRad Ready Gels (SDS-PAGE, 12% and 10-20% linear gradient, Biorad). Separated proteins were transferred electrophoretically to nitrocellulose membrane using a BioRad Mini Trans Blot Electrophoretic Transfer Cell. The transfer of protein from the gel to the membrane was carried out at 100 volts for 1 hour in cold (4°C) transfer buffer (20% methanol, 25 mM Tris, 192 mM glycine) according to the manufacturer's instructions. The transfer buffer was kept at 4°C by an ice insert in the transfer rig. Membranes were generally (unless otherwise specified) blocked with 5% dry milk dissolved in Tris-buffered saline, 0.1% Tween-20 for one hour and incubated with antibody (1/10⁶ dilution of anti-RTGase). Antibody binding was visualized using biotinylated secondary antibody (HRP-linked anti-Rabbit IgG serum, 1/1000) and horseradish peroxidase luminescence with film detection (Hyperfilm from Amersham Life Sciences, New England Biolabs Phototope-HRP Western Blot Detection Kit). Biotinylated markers were visualized with HRP-linked anti-biotin serum (1/1000). Membranes that had been incubated with primary antibody, biotinylated secondary antibody and/or anti-biotin were washed for 1 minute with the substrate and the light production was captured by film exposure to the membrane/substrate. The films were processed by a Kodak M35A X-OMAT Processor.

II.B.14. Recombinant expression

The full length cDNAs encoding the Onmy-Chit.01 and Onmy-Chit.02 chitinases were amplified by PCR from reverse transcribed RNA. Gene specific primers were designed to eliminate the termination codon and to insert an AgeI restriction site. PCR

amplification was performed using the Advantage 2 PCR kit (BD Biosciences, Palo Alto, CA). Each amplicon was ligated into a pDrive vector (Qiagen, Valencia, CA), released by EcoRI and AgeI restriction digest, and subcloned into a similarly digested pMT/V5-His (pMT) expression vector (DES kit, Invitrogen, Carlsbad, CA) thereby generating an in-frame hexahistidinyI fusion (His-tag) at the C-terminus. The control construct is the same vector ligated with green fluorescent protein (GFP), the full length of GFP cDNA is 714bp, and the protein is 26.8kDa (a gift from Nilli Zomra). Transfection, selection, maintenance and induction of expression of the Schneider's S2 cell line were performed according to the manufacturer's protocol (Drosophila Expression System, DES, Invitrogen). Described in brief, the construct is co-transfected with a pCoBlast (Invitrogen) selection vector into S2 cells using Effectene transfection reagent (Qiagen). GFP construct was used as a marker to indicate the stable transfection, which can be examined under UV light. Transfected cells were selected for a period of two weeks in Schneider Insect Medium (Invitrogen) supplemented with Blasticidin S (25 mg/ml) (Invitrogen) and 10% fetal calf serum (FCS). After selection, the cells are transferred to a baffled shake flask in complete medium containing a reduced concentration of Blasticidine (10 mg/ml) and grown to a density of $20\text{-}30 \times 10^6$ cells/ml in a volume of 500 ml. At this point, the complete medium was replaced with 1 liter of serum-free, antibiotic-free medium (complete medium lacking the FCS and Blasticidin). After an adaptation period of 3 days (cells density of 15×10^6 cells/ml), protein expression was induced by the addition of CuSO₄ (500 mM) for 4 days. The medium was harvested and clarified by centrifugation.

II.B.15. Fluorescent *In situ* Hybridization and Immunohistochemistry

Localization

Tissues were taken from rainbow trout and immediately fixed in 4% formaldehyde in PBS. Fixed tissues were dehydrated in ethanol and xylene. After dehydration, tissues were imbedded in paraffin. *In situ* hybridization sections at 6 µm were deparaffinated in xylenes, rehydrated in a graded ethanol series, incubated with 0.2 M of HCl for 20 min, washed for 5 min in PBS, treated with proteinase K (10 µg/ml in 50 mM Tris-HCl, pH 7.5 and 50 mM EDTA) for 15 min and acetylated in 0.1 M triethanolamine-HCl /0.25 % (v/v) acetic anhydride. For pre-hybridization, each section was covered with 500 µl of hybridization buffer II (50% formamide, 5x SSC, 50 µg/ml yeast tRNA and 50µg/ml denatured calf thymus DNA) and incubated for 2 hours at 58°C. After prehybridization, buffer was replaced with new hybridization buffer II containing 400 ng/ml of DIG-labeled denatured probes (Figure II- 2), covered with a cover-slip and incubated overnight at 58°C. Probes are the same as for Northern blotting. After hybridization, the sections are washed for 30 min in 2x SSC at 25°C, 1 hour in 2x SSC at 65°C, 1 hour in 0.4x SSC at 65°C, 1 hour in 0.1x SSC at 65°C and equilibrated for 10 min in buffer I (100 mM Tris-HCl and 150 mM NaCl, pH 7.5). AP-coupled anti-DIG antibody and peroxidase (POD)-coupled anti-FLU antibody (Roche) are applied at 150 mU/ml of each in buffer I. Excess antibodies are removed by two 15 min washes with buffer TNT (150mM NaCl, 100mM 1M Tris pH 7.5 and 0.05% Tween 20). Biotinyl Tyramide Working Solution (PerkinElmer) was used to increase fluorescence signals by incubation in sections for 5 min, and subsequently wash three times with TNT washing buffer for 5 min. The sections were then incubated with streptavidin-fluorescein (Perkin Elmer) substrate for 30 min at

room temp and washed with TNT wash buffer. After detection using a fluorescence microscope, the second staining was applied, using HNPP/ Fast Red Mix (Roche) as fluorescence substrate. Following washes, the sections were mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories) and examined under a confocal microscope.

For immunohistochemistry, sections (6 μm) were deparaffinated in xylenes, rehydrated in a graded ethanol series, incubated with 0.5% H_2O_2 in PBS for 30 min, washed for 5 min in PBS and incubated with 0.5% Triton X-100 in PBS for 8 min. After incubating with blocking buffer (Roche) for 3 hrs, primary antibody is applied and incubated overnight at 4°C , after washing with PBS, biotinylated anti-rabbit IgG antibody (1:200 in blocking buffer) (Vector Laboratories) is applied. The ABC reagent (Vector Laboratories) was used to amplify the signal after washing the secondary antibody. Color development in sections was executed with 3, 3-diaminobenzidine tetrahydrochloride (DAB; Invitrogen). The sections are mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories), and examined under the BH-2 light microscope (Olympus, Japan) and computer images were obtained with a MDS digital camera (Kodak).

II.B.16. Cell fractionation

Fresh extracted rainbow trout blood was centrifuged at 1500 rpm/ 4°C for 10 mins. Buffy-coat cells were washed and resuspended in ice-cold 0.9x PBS plus 5% FBS and adjusted to concentration 1×10^8 cells. Propidium iodide (Sigma) was added to a concentration of 1 $\mu\text{g}/\text{ml}$ to exclude dead cells and debris. Flow cytometry analysis and sorting was based on propidium iodide exclusion, forward scatter (granule) and side

(size) scatter. The operation was done using a FACSVantage SE (Becton Dickinson) at the University of Maryland Baltimore Cancer Center. Cytospin preparations were made with $2 \sim 10 \times 10^5$ cells cytocentrifuged at 300 rpm for 1~3 min onto glass slides on Cytospin2 cytocentrifuge (Shandon). Cytospin preparations were processed through protocol stains (Fisher Sciences), which is similar to wright-giemsa stain, for morphological analyses and observed microscopically using a Nomarski Optics (Zeiss). Cell suspensions were washed and resuspended in cold HBSS. Percoll (Pharmacia) was prepared from stock by adding 1/10 of a volume of sterile 10x PBS (19 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 81 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.37 M NaCl, and 26 mM KCl; pH 7.4) to a density of 1.13 g/ml. Percoll gradients are prepared in the range of 45–65% with HBSS as diluents in 15-ml Falcon tubes with a maximum of seven 2-ml layers. Freshly prepared gradients were allowed to equilibrate for 30 min on ice. Two milliliters of each cell suspension (2×10^8 cells) were layered carefully on top of each gradient, and the tubes were centrifuged for 30 min at $1900 \times g$ at 4°C . Each layer was removed carefully by pipetting out and washed in 10 ml of ice-cold HBSS two times. Cell samples from each layer were stained with trypan blue to determine cell viability and counted using a hemacytometer.

II.B.17. Phylogenetic analysis

Maximum likelihood tree of vertebrate chitinase based on a 1484 bp cDNA sequence alignment, using PAUP*4.0 (Sinauer Associates, Inc.). The general time reversible model was used with invariant site correction and a gamma distributed rate parameter with four categories. The hagfish presumptive chitinase was used as the outgroup.

II.C. Results

II.C.1. Amplification of Rainbow Trout Chitinases and Pepsinogen

A 550 bp sequence with high similarity to mammalian chitinases was successfully obtained using degenerate primers (Figure II- 1A) to rainbow trout stomach cDNA library. The smaller PCR products sequences contained within the larger 550 bp sequence. Based on this 550 bp, we designed 5' and 3' RACE primers and obtained full-length cDNA sequences for Onmy-Chit.01 from the rainbow trout stomach 5' and 3' RACE library amplifications. Using a similar approach based on the partial cDNA obtained from an LPS stimulated macrophage cDNA library [128] the full length cDNA from Onmy-Chit.02 was obtained from a rainbow trout head kidney 5' and 3' RACE library.

The Onmy-Chit.01 cDNA (Figure II- 1B) was 1655 bp in length with an open reading frame (ORF) coding for a 481 amino-acid protein (GenBank accession no. EU877960). The Onmy-Chit.02 cDNA (Figure II- 1C) represented a slightly larger 1931 bp transcript with a coding potential for a 464 amino-acid peptide (GenBank accession no. EU877961). A comparison of the two cDNAs reveals that Onmy-Chit.02 has a longer 5' & 3'UTR, 494 bp for 3' UTR and 48 bp for 5'UTR, compared with 179bp for 3'UTR and 14 bp for 5'UTR of Onmy-Chit.01. Onmy-Chit.01 & 02 share a 56% amino acid sequence similarity. See the amino acid sequence alignment in Figure II- 3 with important structural motifs designated for both proteins.

As with other vertebrate chitinases, the predicted amino acid sequence from Onmy-Chit.01 & Onmy-Chit.02 has four domains (Figure II- 3). They are the signal peptide, catalytic domain, linker region and chitin-binding domain. Protein sequencing on purified

gastric Onmy-Chit.01. The twenty-six N-terminal amino acid residues of the purified native Onmy-Chit.01 protein are marked on the putative amino acid sequence in Figure II- 3. One hundred percent concordance with predicted amino acid sequence was observed indicating the signal peptidase cleaves at the serine residue at position 21 for Onmy-Chit.01.

A rainbow trout pepsinogen cDNA (Figure II- 4A) was successfully obtained from the rainbow trout stomach 5' and 3' RACE library amplifications to provide a cDNA with a total length 1354bp and a coding potential for a 378 amino-acid protein (GenBank accession no. EU880230). The deduced protein sequence of rainbow trout pepsinogen showed significant similarities with other vertebrate aspartic acid proteases (Figure II- 4B) with similarities to pig pepsinogen (53.6%), human pepsinogen A (54.9%), and *Xenopus laevis* pepsinogens (54.8%), respectively (Figure II- 4B).

II.C.2. Heterologous Expression in *Drosophila* S2 Cells

Plasmid constructs containing ORFs of Onmy-Chit.01 & 02 were generated and transfected into the *Drosophila* S2 expression system. Two weeks after blasticidin selection, copper sulfate was added to induce protein production. Cells and media were harvested at day 2 & 5 after induction. Cell extracts and media were tested by western blotting for chitinase expression. No detectable bands were observed in media (data not shown). In cell extracts, the control GFP insert showed two bands when probed with the anti-chitinase polyclonal antibody (Figure II- 5, lane 1,4,6). Onmy Chit.01 (Figure II- 5, lane 3,7) & 02 (Figure II- 5, lane 2,5) stably transfected S2 cell lines exhibited an additional band around 50KDa, which is consistent with uncleaved chitinase protein still

retaining its signal peptide. However, neither cell lysate nor media had detectable chitinase activity.

II.C.3. Chitinase Activity Tissue Distribution

The highest chitinase activity was observed in the stomach ($2173.2 \pm 554.2 \mu\text{g GlcNAc/hr} \cdot \text{mg}$) (Figure II- 6A). In non-digestive tissues, there was little activity, with the exception of slight chitinase activity in the kidney ($59.1 \mu\text{g NAG/hr} \cdot \text{mg}$). In head kidney, detectable activity was only found at pH 7.5 (Figure II- 6A).

II.C.4. Quantitative mRNA Tissue Distribution

We examined Onmy-Chit.01 & 02's mRNA expression levels in various tissues from rainbow trout using quantitative PCR. The gastric portion of the intestinal tract was dissected into three parts based on morphology- cardiac portion, blind sac, and pyloric caeca [20]. As shown in Figure II- 6B, Onmy-Chit.01 mRNA was primarily expressed in the cardiac portion and blind sac of the stomach. Onmy-Chit.02's expression levels were much lower than Onmy-Chit.01 (nearly 26 fold less), but were more widely distributed in expression. Highest expression for Onmy-Chit.02 is found in the spleen, liver and kidney. The qPCR results were consistent with the Northern blot analysis (next section) and chitinase activity analysis described earlier (Figure II- 6A).

II.C.5. Northern Blotting Analysis

A Northern blot analysis using 10 μg of enriched poly-A⁺ mRNA from gill, stomach, peripheral blood, spleen, kidney, head kidney, liver and muscle tissue probed for Onmy-Chit.01 & 02 is presented in Figure II- 7. Probe positions are illustrated in

Figure II- 2. Onmy-Chit.01 was detected in stomach and kidney mRNA pools only; however, Onmy-Chit.02 could be detected in all the examined tissues except muscle (Figure II- 7B). A single band for both chitinases was detected and consistent with the predicted size from the cloned cDNAs. Hence we infer for these adult tissues there is no evidence for alternatively spliced transcripts.

II.C.6. Cellular Distribution of Chitinase Expression

Using fluorescent *in situ* hybridization (ISH) we probed for Onmy-Chit.01 (Figure II- 8A) and rainbow trout pepsinogen (Figure II- 8B) mRNA specific sequences on the histology slides from the cardiac portion, blind sac, pyloric caeca portion of stomach and anterior part of intestine. The sense-probe was used as a non specific negative control for hybridization. As shown in Figure II- 8, Onmy-Chit.01 and rainbow trout pepsinogen mRNA have identical cellular locations, both are expressed in the gastric gland in rainbow trout [129]. No signal could be detected in parallel negative control samples (Figure II- 8C). No signal can be detected in pyloric caeca portion of stomach and anterior part of intestine. To investigate specific localization of the chitinase protein, immunohistochemistry studies were done on parallel slides of the cardiac portion, blind sac, pyloric portion of stomach and anterior part of intestine using the anti-RTGase and anti-progastricsin antibodies (Figure II- 9 A-C). Pre-immune serum from the anti-Onmy-Chit.01 production was used as negative control (Figure II- 9D). Consistent with the FISH results, Onmy-Chit.01 protein was synthesized in the Chief cells of the gastric gland of rainbow trout (Figure II- 9A& B) and secreted chitinase accumulated in the mucosal surface of stomach (see Figure II- 9B). Pro-gastricism protein was detected in the same regions and cells of the stomach (Figure II- 9C). Similar to pepsinogen mRNA

expression, chitinase and progastricsin proteins have identical expression pattern in the stomach of the rainbow trout. The specificity of the immunohistochemistry is evident in lack of staining using pre-immune serum (Figure II- 9D).

Unfortunately, no signal (ISH or IHC) was obtained above background in any tissues expressing high levels of Omny_Chit.02. Accordingly, to increase our signal to noise level for Omny_Chit.02 we next enriched potential cells likely to express this gene and protein (see below).

II.C.7. Cell Sorting and Characterization of Immune Cells

As shown in Figure II- 8, distinct cell populations for rainbow trout peripheral blood buffy-coat can be distinguished using different gates in flow cytometry. Four gates population were collected, $FSC^{hi}SSC^{hi}$, $FSC^{int}SSC^{int}$, FSC^{lo} and $FSC^{int}SSC^{lo}$. Based on zebrafish cell lineage flow cytometry distribution[130], Myelomonocytic cells (including neutrophils and macrophage) are in $FSC^{hi}SSC^{hi}$ & $FSC^{int}SSC^{int}$ population; lymphoid cells are in a $FSC^{int}SSC^{lo}$ fraction; mature red blood cells are exclusively in a FSC^{lo} population, and immature precursors are in a $FSC^{hi}SSC^{int}$ subset.

Cytospin slides for cells collected from the above four gates were made and stained. Within these gates, five cell types of rainbow trout blood were easily distinguished from the staining patterns. As shown in Figure II- 10, red blood cells are in the left upper corner, which are characterized by their elliptical shape with pink cytoplasm and dark blue nucleus; macrophages are in right upper corner, which characterized by having large sky-blue cytoplasm and a violet nucleus; neutrophils are also in right upper corner have a pale pink cytoplasm and multi-lobe shaped nucleus; monocytes are in left lower corner with a dark blue cytoplasm and violet nucleus; and lymphocytes are in the right lower

corner, are smaller than macrophages and neutrophils, with a dark blue cytoplasm and huge violet nucleus. In our samples we were unable to detect thrombocytes.

II.C.8. Percoll Enrichment of Immune Cells

Four layers of cells were collected from percoll-separated head-kidney cells, Figure II- 11 shows random snapshots of stained cytopsin slides of these cell layers. These data suggest that layer HK P45 mainly contain monocytes, some neutrophils and macrophages, few lymphocytes; layer HK P50 primarily contains neutrophils, some macrophages and few lymphocytes; layer HK P55 has about equal percentage of neutrophils, macrophages and lymphocytes while layer HK P60 contains mainly lymphocytes, few neutrophils and macrophages.

II.C.9. Quantitative Real-time PCR of Onmy-Chit.02 Transcripts with Percoll Separated Cell

Real-time qPCR tests on Onmy-Chit.02 were performed on the above four layers and buffy-coat cells from head kidney and peripheral blood. The HK P50 fraction exhibited the highest Onmy-Chit.02 expression levels with HK P45 having the lowest expression (Figure II- 12). Human chitotriosidase was only expressed in macrophages, but not in monocytes. It seems that Onmy-Chit.02 does the same.

In mammalian systems, neither chitinases are expressed in lymphocytes. However, rainbow trout lymphocytes may express Onmy-Chit.02. Layer HK P60 contains few myeloid cells, however, Onmy-Chit.02 expression level is just slightly lower than HK P55, which contains an equal percentage of neutrophils, macrophages and lymphocytes. If rainbow trout lymphocytes do not express Onmy-Chit.02 at all, expression level lower

than head kidney buffy-coat should be seen in Layer HK P60 as seen in layer HK P45, since there are just a few macrophages and neutrophils. In contrast chitinase expression level of HK P60 is much higher than buffy-coat layer, which could indicate that rainbow trout lymphocytes may indeed express Onmy-Chit.02.

II.D. Discussion

We successfully cloned two distinct full-length chitinase cDNAs from rainbow trout that exhibit wide differences in tissue specificities and expression levels. Both appear to code for secreted proteins with the classic vertebrate chitinase bipartite structural motifs[40], an N- terminal catalytic domain followed by a linker region to a C-terminal chitin-binding domain. Starting with amino acid residue 22 of the Onmy-Chit.01 predicated protein sequence, the next 23 amino acids match in perfect concordance with the Edman degradation products for the purified Onmy-Chit.01 protein[131] (Figure II-3). This suggests that the rainbow trout signal peptidase cleaves at the serine (residue 21) residue of the signal peptide in Onmy-Chit.01.

The Onmy-Chit.01& Chit02 proteins share 56% sequence similarity. While the catalytic activity and substrate specificity of Onmy-Chit.01 have been well characterized [132], similar data for Onmy-Chit.02 is not available. Both of them can be expressed in S2 cell expression system but without enzymatic activity. This could be due to lack of proper post-translational modifications in the Drosophila cell system, such as the signal peptide of the proteins appear not to be cleaved. From previous published data for Onmy-Chit.01[132], we predict that Onmy-Chit.01 encodes a secreted exo-chitinases that releases N-acetyl glucosamine (GlcNAc) dimers from the non-reducing end of a chitin polymer. We expect the enzyme to be specific for the β - (1, 4) linkage in the unbranched chitin homopolymer.

Like chitotriosidase and AMCase in humans, Onmy-Chit.01 & 02 have very different distributions patterns across tissues. Onmy-Chit.01 exhibits extremely high expression and activity in the gastric portion of the stomach presumptively in the gastric gland which

is similar to the human and mouse AMCase cellular expression [92]. Onmy-Chit.01 exhibits extremely low expression in the pyloric caecae of the rainbow trout, a major secretory gland in fish. In both human and mouse AMCase expression has been shown in alveolar macrophages of lung and is exaggerated in the lungs of human asthmatics and mouse models [113, 114]. In rainbow trout, the amount of Onmy-Chit.01 expression in the gill is insignificant and may represent activity observed in the whole blood. This indicates that the chitinase in alveolar macrophages plays a derived function in mammals other than food processing.

In contrast, rainbow trout Onmy-Chit.02 is primarily expressed in immune related organs (Figure II- 6), similarly to human chitotriosidase which is expressed at relatively high levels in lymph node, bone marrow and lungs[92]. Expression of mouse chitotriosidase is confined to the tongue, stomach and brain [106].

However, in cellular distribution, we are not able to classify whether Onmy-Chit.02 is expressed in myeloid cell lineage of rainbow trout, which is the case for human chitotriosidase. Therefore, in regards to tissue expression, Onmy-Chit.02 is more similar to human chitotriosidase, a clear immune cell expression lineage. We are not able to determine whether Onmy-Chit.02 is only expressed in the myeloid cell lineage of rainbow trout immune cells at this time, which is the case for human chitotriosidase [92].

We used Percoll separation to enrich various immune cell populations and quantified Onmy-Chit.02 transcript abundance on both enriched myeloid and lymphoid cells with expression in both pools. Although enriched myeloid cells had significantly ($P<0.05$) higher Onmy-Chit.02 expression level than the enriched lymphoid cells, expression was

still detected in lymphoid cells. One possible reason is contamination of myeloid cells, but it is likely that rainbow trout lymphocytes could also express Onmy-Chit.02.

Chondrichthyes represent the earliest group to obtain adaptive immunity, and it is in a transitional period which some cell types important in innate immunity evolved into highly specialized components of the adaptive immune response in higher vertebrates[133]. B lymphocyte (IgM⁺) in rainbow trout are able to ingest particles and bacteria *in vitro* and *in vivo*, and it may have evolved from ancient phagocytic cells[133]. It is very likely that lysosomes in B cell of rainbow trout share the same enzyme content as macrophages and neutrophils, namely, Onmy-Chit.02 might be expressed by B lymphocytes in rainbow trout.

A phylogenetic analysis of known vertebrate chitinase cDNAs (Figure II- 13) finds them to cluster into four groups. Two groups are composed only by fish chitinases while the two other groups contain the mammalian chitotriosidases and AMCases. Onmy-Chit.01 & 02 reside separately in the first two groups. Onmy-Chit.01 groups with striped bass gastric chitinase and flounder fchi1&fchi2 which are known to be expressed in the stomach[120, 132]; Onmy-Chit.02, however, groups with the chitinases found in the genomes of the classic teleost fish models (zebrafish and pufferfish) which are known as stomachless fish [134]. Their esophagus directly connects to intestine, and the gastric portion is absent. In these stomachless fish, it is very common that enzymes typical of the stomach are expressed in other tissues. In pufferfish, where all five types of digestive aspartic proteinases exist in its genome, pepsinogen expresses in skin; nothepsin in the liver, ovary and testis; rennin in the spleen, kidney and testis etc. [134].

A difference in expression location of these two rainbow trout chitinases may underlie a new adaptation of their function. In the case of chitinase, Onmy-Chit.02 has similar cellular distribution similar to human chitotriosidase, however, phylogenetically, they are far apart, which indicate that Onmy-Chit.02 and chitotriosidase could also be generated after speciation. Although Onmy-Chit.01 group is a fish group, AMCase group is closer to Onmy-Chit.01 group than to chitotriosidase group. Onmy-Chit.02 is distantly related to all three other clades, but closer to the root- hagfish chitinase, which could indicate that Onmy-Chit.02 clade is more primitive. We infer from our cellular distribution findings that this indicates its role in the immune system is basal.

In mammals, AMCase is expressed in alveolar macrophages, which is elevated in asthma. It is very likely that AMCase is secreted by alveolar macrophages to clear up invading chitin-containing pathogens. From this point of view, large amounts of chitinase in rainbow trout stomach could also functions in removing chitin-containing pathogen. Cold-water fish, like rainbow trout, do not have long curved digestive tract and hence food sits in the stomach for a long time [135]. In addition, its stomach pH is higher than mammals, usually around pH 4.5 instead of pH 2. Pathogens have much greater chance to survive in the cold-water fish stomach than in the mammals. Stomach chitinase may play an important role in defending against these pathogens in poikilotherms. This hypothesis matches with our presumption that chitinase's role in the immune system is basal. In order to adapt to new environment, Onmy-Chit.01 and AMCase went through mutational changes which permit a lower pH optimum and resistance to stomach proteases, which explains the differences between amino acid sequences of immune chitinase and non-immune chitinase.

However, stomach chitinase's role in immunity should be secondary compared to food processing since some herbivorous fish, such as carp, do not show chitinase activity in their stomach[88]. These fish also encounter challenges of chitin-containing pathogen from food. Non-detectable chitinase activity could mean either chitinase is not the strategy they use to fight with these pathogens, or chitinase can be induced upon infection but not constitutively expressed. Further experimentation is needed to solve this puzzle.

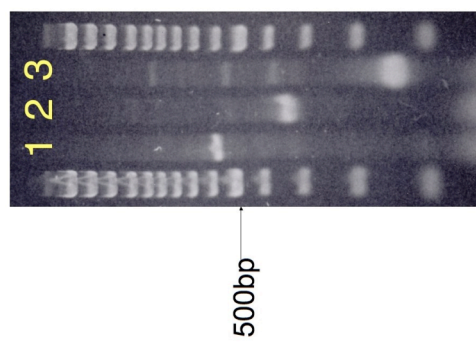
An evolutionary birth and death model [136] fits chitinase gene family very well. The two chitinase genes in rainbow trout derive from duplication of common ancestor chitinase gene. The genes have undergone further duplication and may occasionally lose its chitinolytic activity or generate pseduogenes, thus we have many chitinase-like proteins, such as human y39Kd, mouse Ym1 etc, phylogenetically which are close to chitotriosidase.

Human chitotriosidase and AMcase genes have very different promoter regions as do human and mouse chitotriosidase genes[92]. Perhaps comparison of three genes' promoter regions will help to understand evolutionary relationship of those enzymes and also shed a light on chitinase regulatory mechanism.

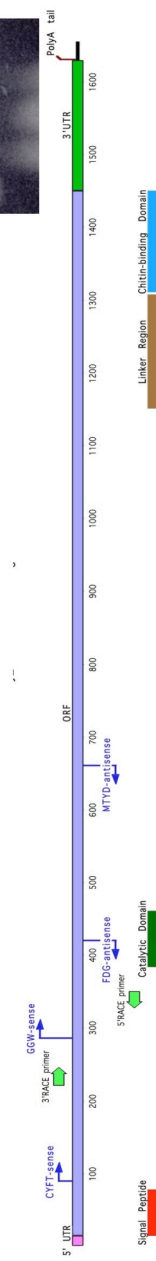
Figure II-1. Cloning of rainbow trout (*Oncorhynchus mykiss*) chitinases. Three degenerate primer sets (inset) were designed based on alignments of vertebrate chitinases. An aliquot of 100 ng of the first-strand from a stomach cDNA library was amplified with the degenerate primer pairs and separated on a 1% agarose gel (A). Based on the sequences obtained 5' and 3' RACE primers were generated and used to obtain a full length Onmy-Chit.01 cDNA of 1655 bp (B). The location of important structural motifs are mapped on the drawing. From an LPS induced cDNA library from rainbow trout macrophages [19] a partial chitinase transcript was isolated and extended using 5' and 3' RACE. A Onmy-Chit.02 full length cDNA of 1931 bp was obtained (C). The location of important structural motifs are mapped on the drawing as above for Onmy-Chit.01.

A:

Reaction	Forward Primer	Reverse Primer	Annealing Temp. (°C)	Band Size (bp)
1	CYFT-sense	MTYD-antisense	40.5	571
2	CYFT-sense	FDG-antisense	48.4	331
3	GGW-sense	FDG-antisense	48.4	134



B:



C:

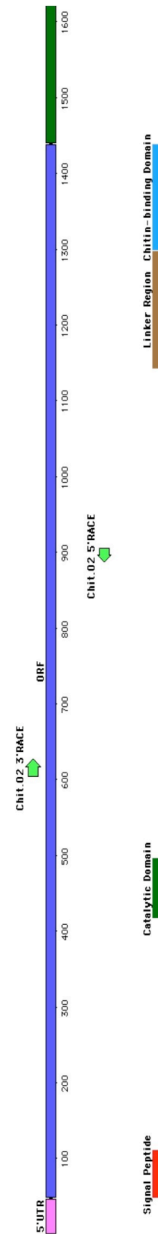
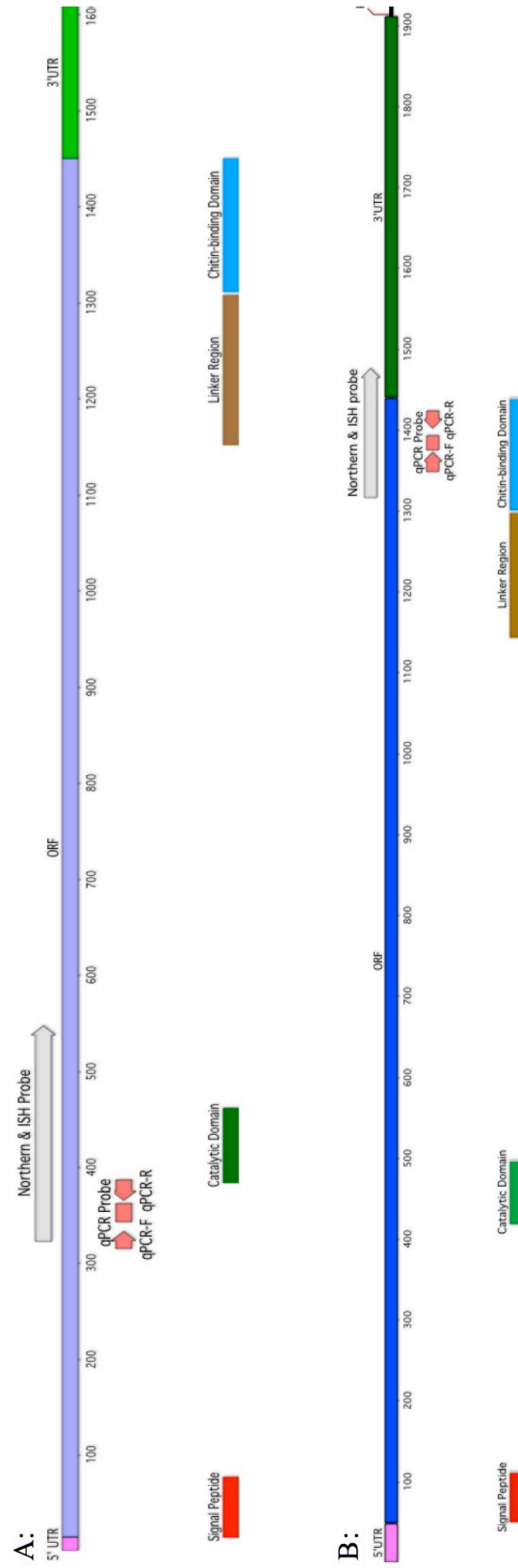


Figure II-2. Primers and probes locations for qPCR, In situ hybridization and northern blotting. The location of important structural motifs are mapped on the drawing. A: Onmy-Chit.01 sequence; B: Onmy-Chit.02 sequence. The probes for northern blotting and in situ hybridization are identical for both genes.

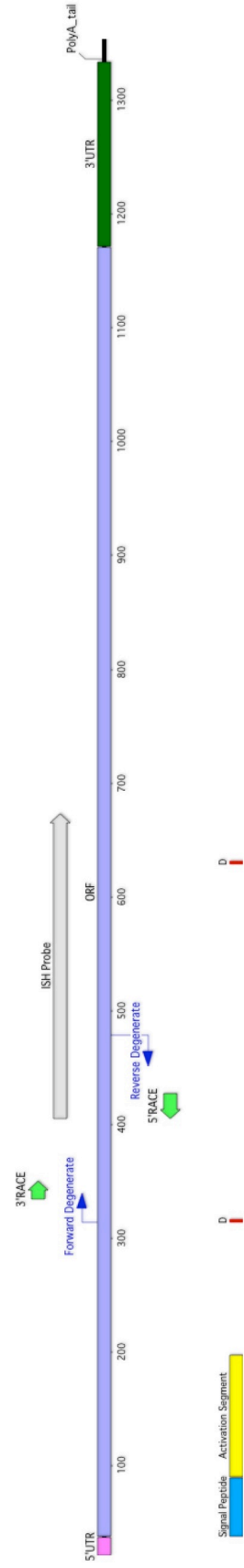


Onmy_CHIT.02	1	MSKLTLAGLCFAL-CQLGSTS	QLVCYFTNWSQYRPGTGKFLPANVDPHL	49
Onmy_CHIT.01	1	MGKLLICVGLALLLHAQLGSS	YILSCYFTNWGQYRPGAGKYFPT	50
		* * * . * * . * * * * . * * * * * * * * . * . * * * * *		
Onmy_CHIT.02	50	CTHLIYAFSIINPANELATYEWNDETLYSSFNGLKDRNPQLKTLLAVGGW		99
Onmy_CHIT.01	51	CDHLIYAFAGMAN-NEIKTYEWDDEKLYGQFQALKNQNSNLKTLLAIGGW		99
		* * * * * . . * * . * * * * * * * * . * * . * . * * * * * *		
Onmy_CHIT.02	100	KFGTQQFSIMVSSPDNRLKFIQSSISFLRRHSF	DGLDLWE	149
Onmy_CHIT.01	100	NFGTQPFAMVSSAANRQTFISSVIKFLRQYQF	DGLDIDWE	149
		* * * * * . * * * * * * * * * * * * . * * * * . * * * * * *		
Onmy_CHIT.02	150	EDKQRFTLLCKELLEAFEAEKAVSRPRLLLTAAVAAGKGTIDSGYEIAE		199
Onmy_CHIT.01	150	ADKARFTTLLQELMAAFEAEKNTNLPRLMLTAAVAAGKGTIDTGYQIAE		199
		* * * * * . * * . * * * * * * * * * . * * * * * * * * * * *		
Onmy_CHIT.02	200	IAKYLDFISVMTYDFHGSWETFTGHNSPLYQGSHDTGDHIYLNDFAMKY		249
Onmy_CHIT.01	200	IGSVLDYLHVMTYDFHGSWEHNTGENSPLYRGPADQGDYIYFDVDYAMKY		249
		* * * . . * . * * * *		
Onmy_CHIT.02	250	WRDQGAPVEKLMMGFATYGRSFQLAS-VDSGVGASANGAAAAGPFTREAG		298
Onmy_CHIT.01	250	WKSSGAPAEKLLVGFPPTYGHTFQLASGSNTGVGAPATGPGPAGPFTRQSG		299
		* . * * * * * * . * * * * * . * * * * * . * * * * * * * . * * * * * . *		
Onmy_CHIT.02	299	FWSYYEICTFL-QGASFQWIEDQKVPYAS-KGNQWVGFDNRESYDTKVG		346
Onmy_CHIT.01	300	FLAYYEICTLLKQGATQAWDSAQMVPYAYTQQNIWVGVDNIKSQYQDKIEW		349
		* . * * * * * * * * * * . * * * * * . * * * * * * * * * * *		
Onmy_CHIT.02	347	LKENGFGGAMVWNLDLDFAGQSCGQGNYP	LISHLQKLLNIER-----	389
Onmy_CHIT.01	350	LKNTGFGGAMVWSLDLDFSGTFCGQGRYP	LINTLKSGLGTGAGCAARTG	399
		* * . * * * * * * * * * * . * * * * * * * * * * . * . *		
Onmy_CHIT.02	390	--PPLPPTHTPMPGEPP---TVKATTKASGSFCAGRADGLYVKADSPSS		433
Onmy_CHIT.01	400	PIAPVTPAQQLNPQQPGQGSSGSSGSSGSGFCAGKADGMYPDPTNKNS		449
		* . * . . * . . * * * * * . * * * * * * *		
Onmy_CHIT.02	434	FYNCANGITWIQSCPAGLVFSDSCKCCNWP		464
Onmy_CHIT.01	450	FYNCSQGKTYDQHCAAGLVFDISCKCCN		480
		* * * * . * * * * * * * * * * * * * * * * *		

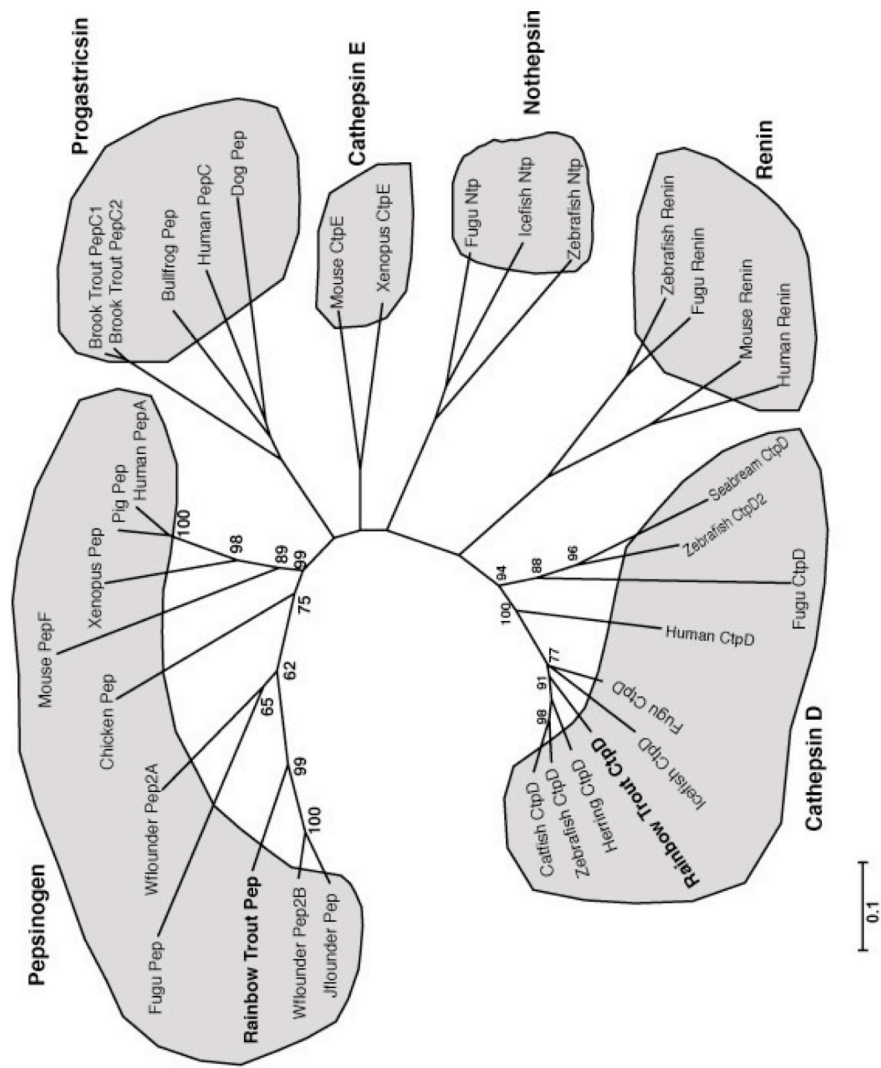
Figure II-3. Amino acid sequence alignment of Onmy-Chit.01 and Onmy-Chit.02. The signal peptide (red), catalytic core (green), linker regions (brown) and chitin binding domain (blue) are indicated. Also yellow shadow marks the amino acid sequence obtained by Edman degradation of the purified Onmy-Chit.01 protein.

Figure II-4. Cloning of rainbow trout (*Oncorhynchus mykiss*) pepsinogen. A degenerate primer set was designed based on alignments of vertebrate pepsinogens. An aliquot of 100 ng of the first-strand from a stomach cDNA library was amplified with the degenerate primer pairs. Based on the sequences obtained 5' and 3' RACE primers were generated and used to obtain a full length pepsinogen of 1354 bp and a coding potential for a 378 amino-acid protein (A). The location of important structural motifs are mapped on the drawing including the catalytic aspartic acid residues (marked as D), location of in situ hybridization probe was indicated too. An unrooted phylogenetic tree is presented for the deduced amino acid sequence showing it mostly closely resembles other vertebrate pepsinogens (B).

A:



B:



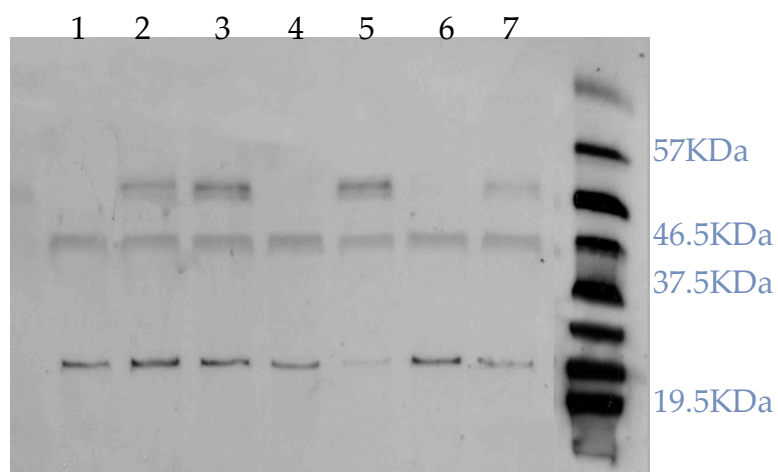
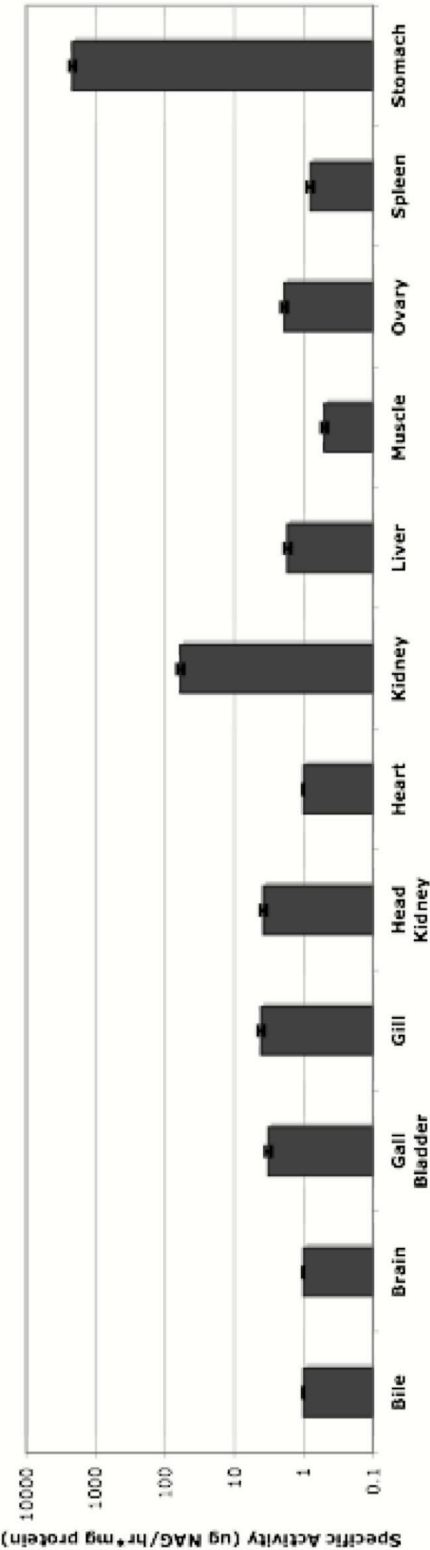


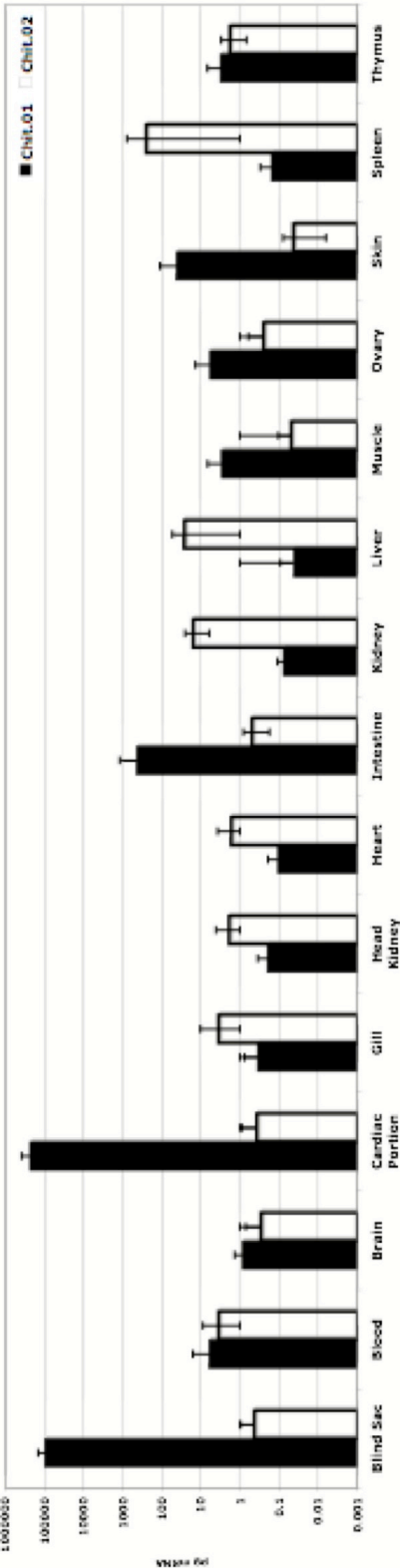
Figure II-5. Western blot of stable transfected *Drosophila* S2 cell expressing Onmy-Chit.01 and Onmy-Chit.02. Lanes 1, 4 and 6 are transfected lines expressing the control insert. Lanes 3 and 7 are transfected lines expressing Onmy-Chit.01. Lanes 2 and 5 are transfected lines expressing Onmy-Chit.02. No chitinase activity or antibody detectable material was observed in the culture media.

Figure II-6. Rainbow trout tissue distribution of chitinase activity and transcript abundance for Onmy-Chit.01 (Filled bars) and Onmy-Chit.02 (Unfilled bars) using qPCR analysis. (A) Protein activity, head kidney was tested at pH7.5, all other tissues were tested at pH4.5;(B) mRNA expression.

A:



B:



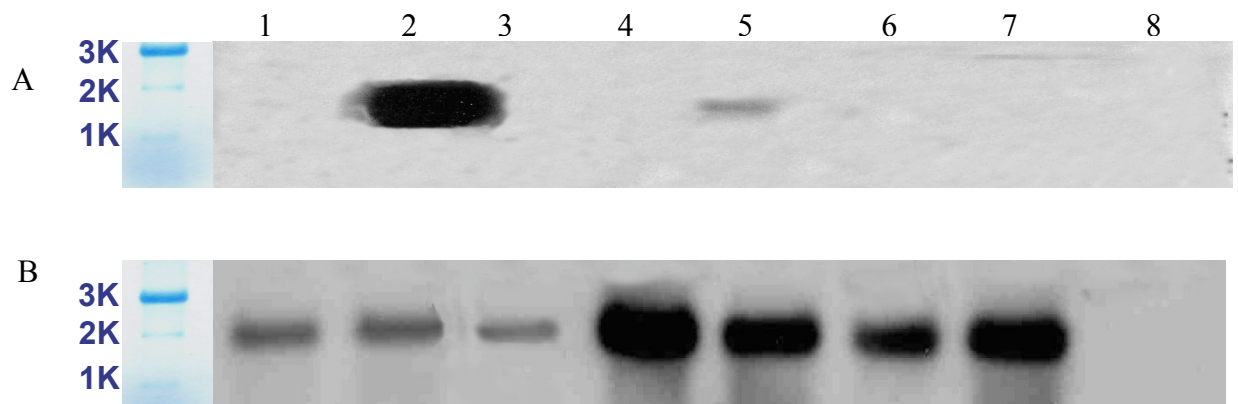


Figure II-7. Northern blot analysis of mRNA enriched total RNA from various organs of rainbow trout. (A) Probed with Onmy-Chit.01. (B) Probed with Omny_Chit.02. Lanes (1) Gill; (2) Stomach; (3) Peripheral Blood; (4) Spleen; (5) Kidney; (6) Head Kidney; (7) Liver; and (8) Muscle.

Figure II-8. Fluorescent *in situ* hybridization for rainbow trout Onmy-Chit.01 and pepsinogen in tissues from the anterior portion of the rainbow trout stomach. (A) using anti-sense ribo-probe of Onmy-Chit.01 with FITC label (B) anti-sense. probe of rainbow trout pepsinogen labeled with DIG and stain using HNPP (C) sense probe for rainbow trout pepsinogen. The section thickness was 6 μ M.

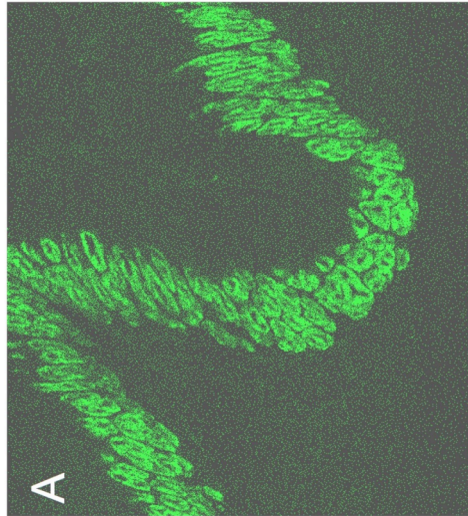
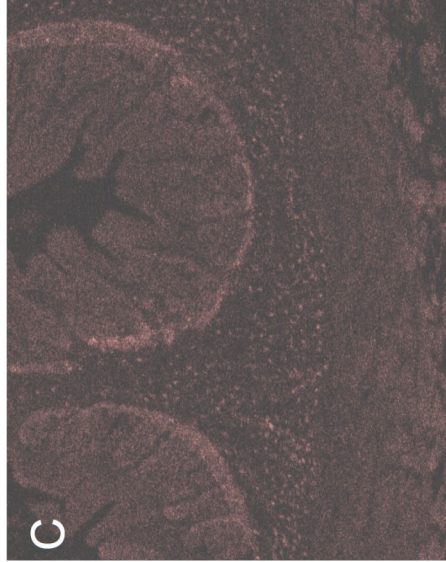
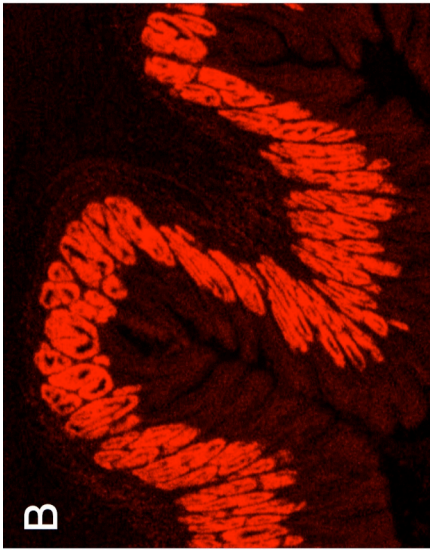


Figure II-9. Immunohistochemistry of cross reacting material for rainbow trout chitinase and progastricsin. (A& B) anti-Onmy-Chit.01 (C) anti-progastricsin and (D) pre-immune serum. The section thickness was 6 μ M.

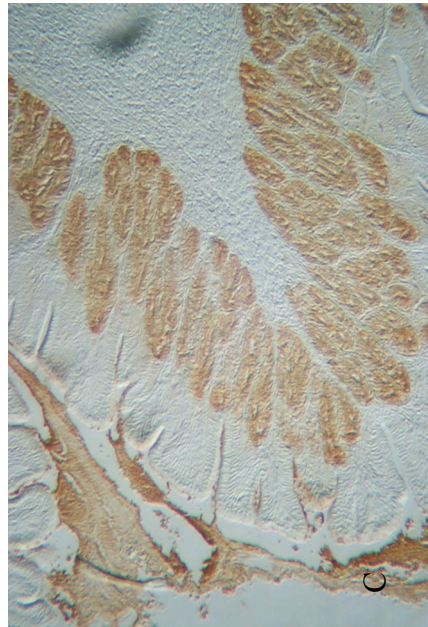
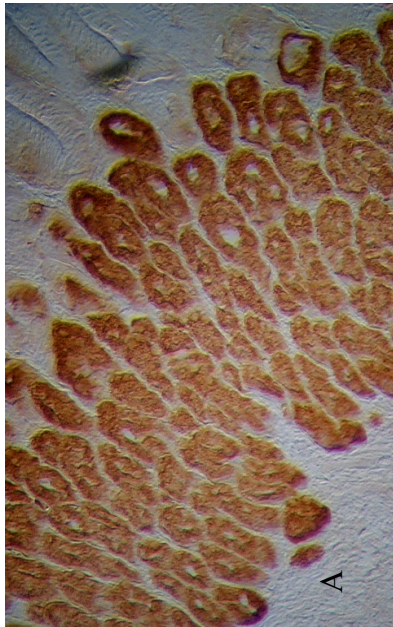
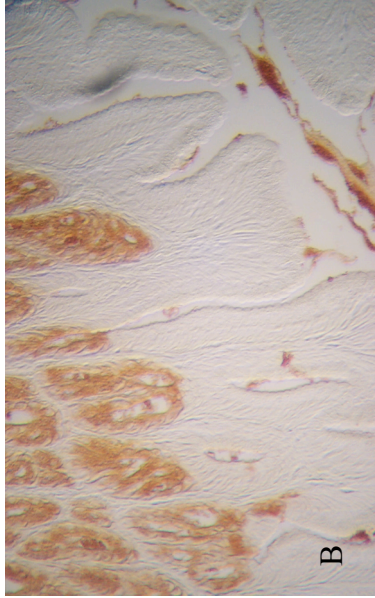
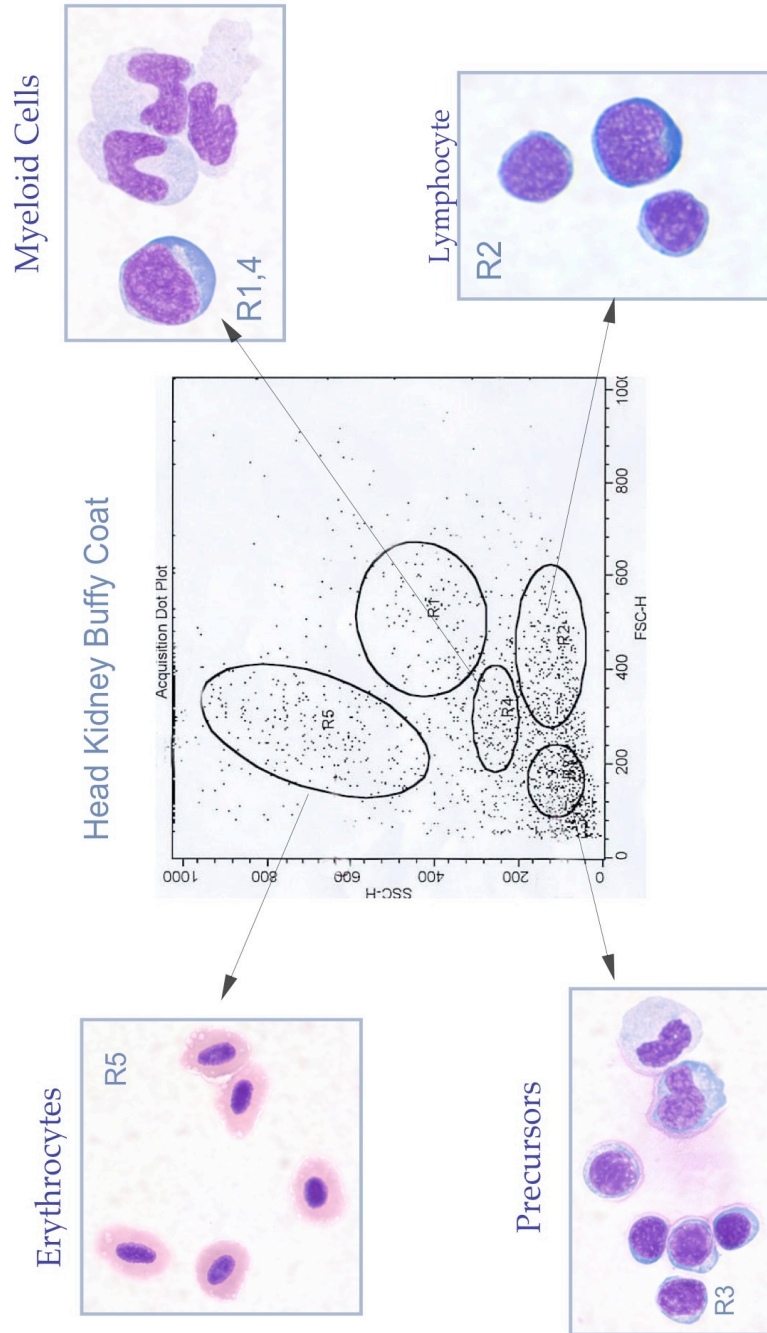
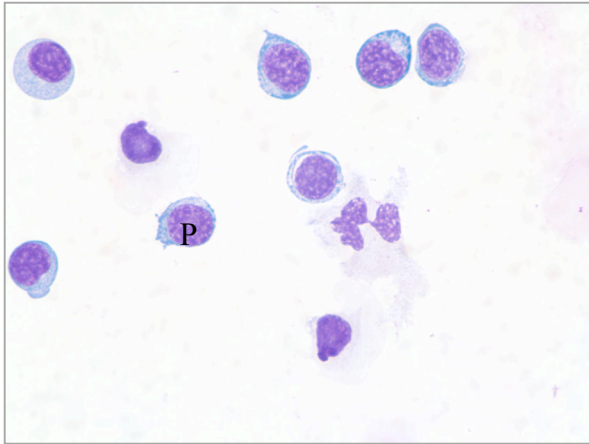
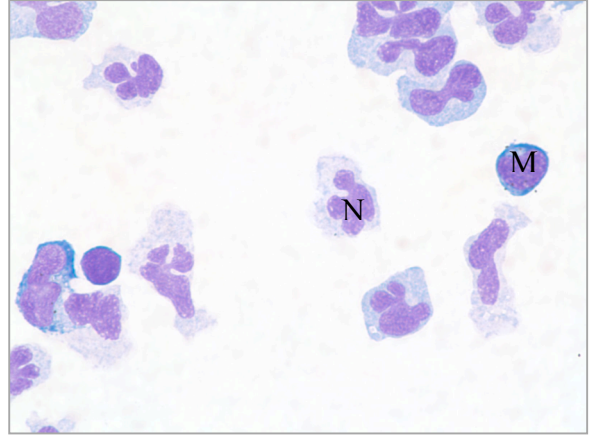


Figure II-10. Flow cytometric separation of rainbow trout blood cells. Suspensions of head kidney cells form distinct populations when analyzed by size (forward scatter; FSC) and granularity (side scatter; SSC). R1&R4 gates contain primarily myeloid cells(upper right panel); R2 gate contains mainly lymphocytes (lower left panel); R5 contains only mature erythrocytes (upper left panel); and R3 gate contains immature precursors of all mature blood lineages (lower right panel).

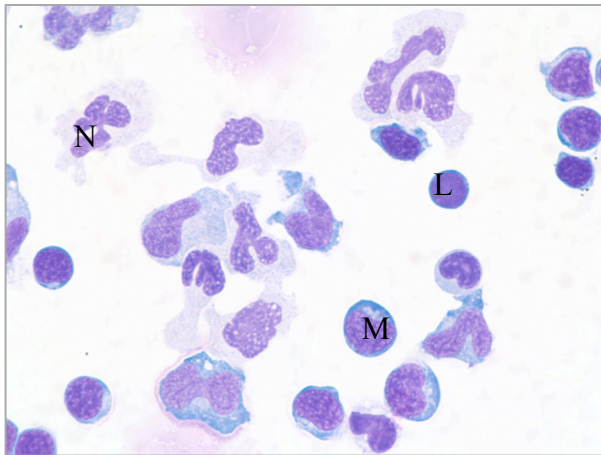




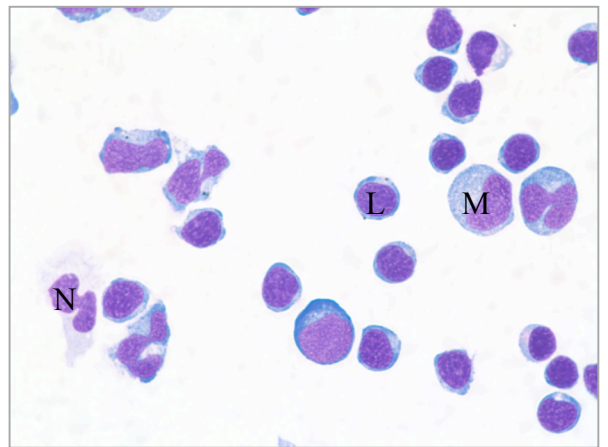
HK P45



HK P50



HK P55



HK P60

Figure II-11. Percoll centrifugation of rainbow trout blood cells. Stained Cytospin slides of percoll separated head kidney buffy-coat cells. HK P45: cells collected at 45% percoll density; HK P50: cells collected at 45% percoll density; HK P55: cells collected at 55% percoll density; HK P60: cells collected at 60% percoll density. N indicates neutrophil; M indicates macrophage and monocytes; L indicates lymphocyte; P indicates precursor cell.

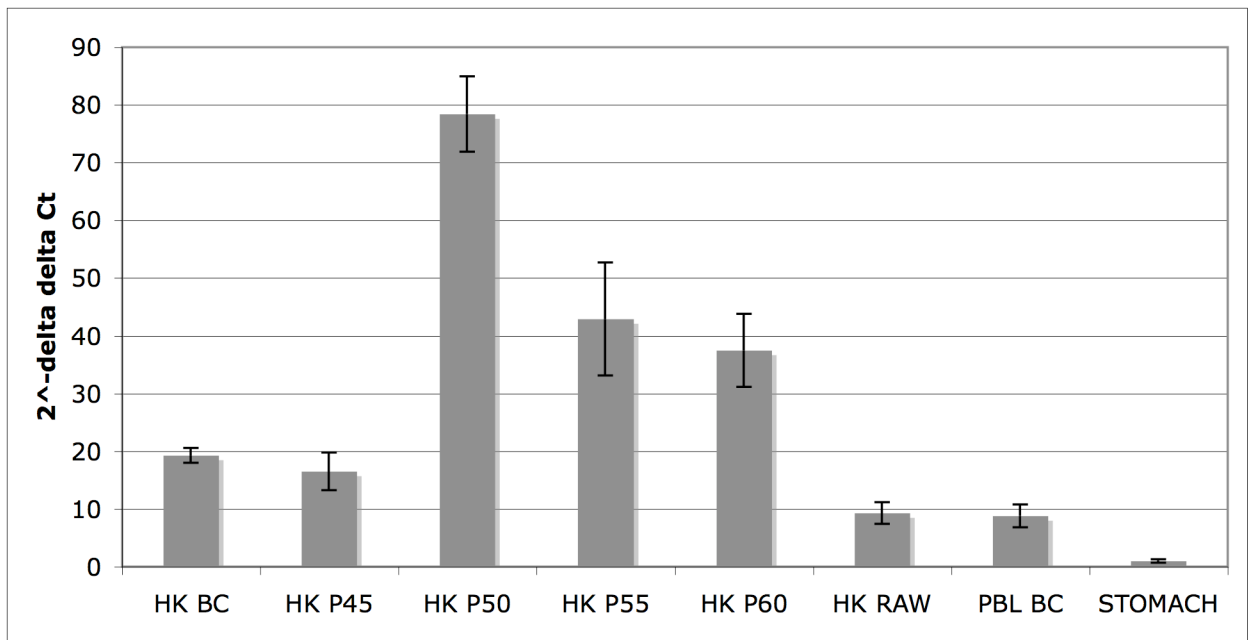


Figure II-12. qPCR analysis of transcript abundance for Onmy-Chit.02 on percoll-separated cells. HK BC: head kidney buffy-coat cells; HK P45: head kidney buffy-coat cells collected at 45% percoll density; HK P50: head kidney buffy-coat cells collected at 50% percoll density; HK P55: head kidney buffy-coat cells collected at 55% percoll density; HK P60: head kidney buffy-coat cells collected at 60% percoll density; HK raw: fresh ground head kidney cells; PBL BC: peripheral blood cell buffy-coat.

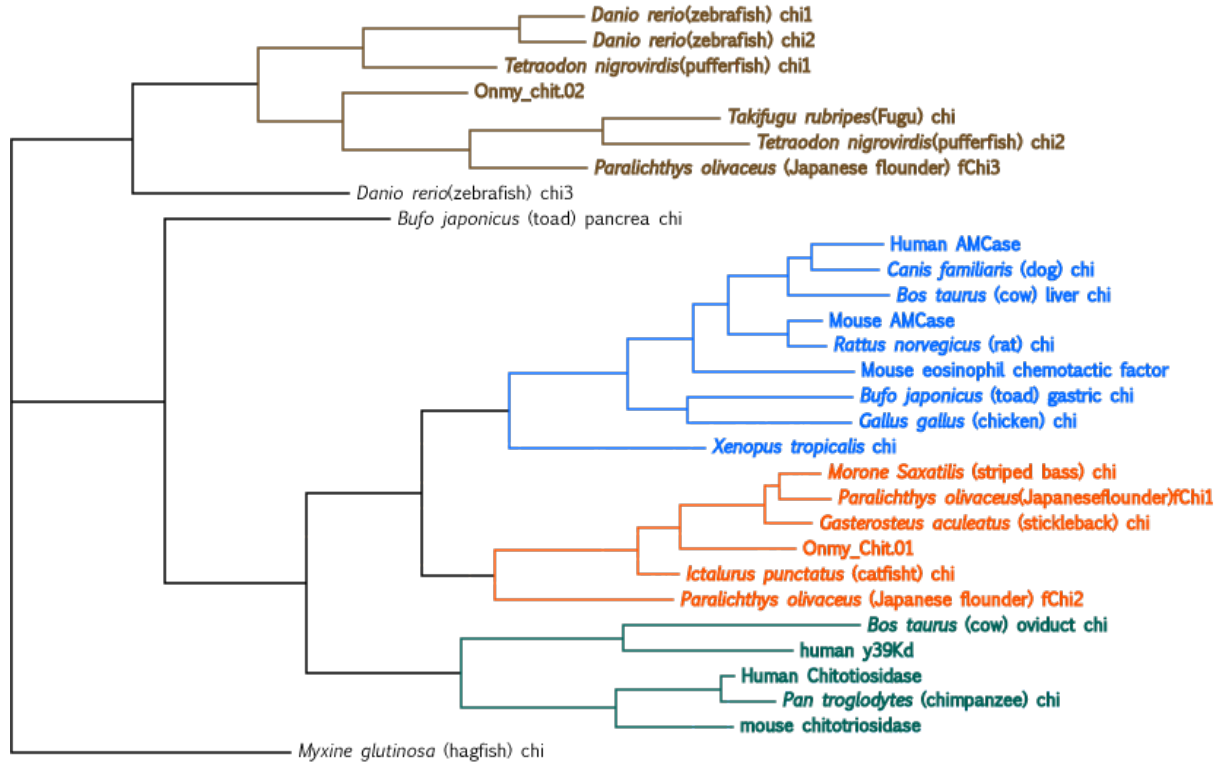


Figure II-13. Phylogenetic tree of known vertebrate chitinases. Maximum likelihood tree of vertebrate chitinase sequences using a 1484 bp alignment. The general time reversible model was used with invariant site correction and a gamma distributed rate parameter with four categories. The hagfish presumptive chitinase was used as an outgroup. Rainbow trout Onmy-Chit.01 groups with other fish gastric chitinases while rainbow trout Onmy-Chit.02 groups with a second class of fish chitinases which would we argue are involved in immune function.

Chapter III The Gene Structure Of Rainbow Trout Chitinases

III.A. Introduction

Recent genomic examinations find two chitinase (E.C 3.2.1.14) orthologs in vertebrates. In human and mice, these two orthologs have very different tissue distribution patterns and are involved in distinct pathological processes. One is called AMCase (acid mammalian chitinase) and expressed in alveolar macrophages and in the stomach of the gastrointestinal tract. The other chitinase named chitotriosidase has a different tissue distribution pattern between human and mouse. In mice, chitotriosidase is expressed in the gastrointestinal tract, the tongue, fore-stomach, kidney, brain, skin, testis and Paneth cells in the small intestine, whereas in human the enzyme is expressed exclusively by professional phagocytes. However, chitotriosidase has never been characterized in non-mammalian animals, though chitinase cDNAs have been cloned or deduced from genomic data. Thus, it is very important to study the evolutionary relationship of two chitinase genes and their regulatory mechanisms, which will help us to understand functions of these two functional similar proteins.

We discussed tissue and cellular distribution of two rainbow trout chitinases in the previous chapter. As we pointed out, Onmy-CHIT.01 & 02 have very different tissue distribution patterns, sharing some common sites of expression with their mammalian equivalents. Both human and mouse AMCase are expressed in gastrointestinal tract and lung, Onmy-CHIT.01 is expressed mainly in stomach and kidney.

Human chitotriosidase is expressed exclusively by professional phagocytes, whereas mouse chitotriosidase is expressed in the gastrointestinal tract, the tongue, fore-stomach,

brain and Paneth cells in the small intestine[92, 106]; Onmy-CHIT.02 is expressed in relatively high amounts in head kidney, kidney, liver, thymus and spleen, furthermore, it may be expressed by both myeloid and lymphoid cells.

Based on a phylogenetic analysis of known vertebrate chitinase sequences we find they cluster into four groups (Figure II-13). Two groups are composed only of fish chitinases sequences, with Onmy-CHIT.01 & 02 residing separately in these two groups. The other two clades are constituted by chitinases from other vertebrates, which span from amphibians to mammals. These two groups can be characterized as an AMCase clade and chitotriosidase clade. The AMCase clade contains both human and mouse AMCase. The chitotriosidase clade includes human and mouse chitotriosidase as well as several other mammalian chitinase-like proteins.

The human AMCase gene is located on chromosome 1p13, whereas the locus of the human chitotriosidase gene is found on chromosome 1q32[137]. Both of mouse chitinases are located in the corresponding syntenic regions, AMCase gene on chromosome 3F3, chitotriosidase on chromosome 1E4[1]. The gene structures (number of introns, etc.) of AMCase and chitotriosidase in human and mouse are very similar. However, the mouse and human chitotriosidase have distinctively different promoters, while the promoter for AMCase genes are relatively similar [1]

Because specification of duplicated genes is considered to be a major driving force for diversity and evolution, it is important to understand the function and regulation of the duplicated genes. The present paper is an attempt to answer the following questions:

1) Are the gene structures in fish and mammalian chitinases similar; 2) Do they show

similar promoter elements; 3) What is the evolutionary origin of these two vertebrate chitinases; and 4) What are the functional relationships of the two chitinases?

The rainbow trout is an ideal model for studying the evolution of chitinase. While the classic teleost models are zebrafish and pufferfish, neither of them contain a functional stomach, which means they should not have an AMCase orthologue[137]. In fact, all zebrafish and pufferfish chitinase genes obtained by genomic blast reside in the Onmy-CHIT.02 group. However, the genome for rainbow trout is not yet available. In order to address this important question, we set out to sequence and assemble BAC clones from rainbow trout containing the chitinase genes. The genomic structure and promoter regions are contrasted with other vertebrate chitinases.

III.B. Material & Methods

III.B.1. BAC library Construction and BAC library screening- in collaboration with Dr. John Hansen

The construction and screening process was performed as described [138, 139].

III.B.2. BAC library screening probe preparation

For the isolation of two the chitinase genes from the BAC clone library, a combination of two probes was used for screening. I tried to obtain single exon probes for both Onmy-CHIT.01 and Onmy-CHIT.02, but I was not able to get any amplicon larger than 200bp from Onmy-CHIT.02. The probe used for Onmy-CHIT.02 bridged through a 88bp intron. Probes were amplified from cloned cDNAs (Figure III-1, blue label), labeled with [P^{32}]-dCTP by random priming (BRL), and then used to screen filters derived from the arrayed OSU and Swanson BAC libraries. Amplified products were cloned into the pTOPO vector to confirm the identity of the amplified fragments using automated DNA sequencing (ABI 3130XL).

III.B.3. BAC plasmid DNA preparation

BACs from two clones (178-C20 & 228-I23) were extracted by using Qiagen large construct kit (Qiagen). Briefly, glycerol stocks from -80° C freezers recovered for 8 hrs in 30°C, and 150µl stock was plated on chloramphenicol (34 µg /µL) containing LB agar plate. After 20 hrs incubation in 37°C, a single colony was selected and inoculated with 3 ml chloramphenicol (34 µg /µL) containing LB medium. The medium was incubated for 16 hrs at 37°C, afterwards, half of the medium was used to inoculate 250 ml

chloramphenicol containing LB medium, and the other half was kept in 15% glycerol stock in -80°C. The plasmid extraction procedures follow manufacturer's instruction. Four plasmids samples from each clone were extracted with average concentration 100ng/μl measured by using a spectrometer.

III.B.4. BAC stability

In order to investigate BAC clones' stability, four plasmids from each clone were digested with three restriction endonucleases (EcoRI, NotI & SalI). The digested BAC plasmids were run on 1% Seaplaque GTG agarose (Cambrex) overnight at the manufacturer recommended conditions (Figure III-2). Pulsed field gel electrophoresis was performed in CHEF Mapper XA system (Bio-Rad). The samples were subjected to PFGE on a 1% agarose gel with 0.5× Tris-borate-EDTA buffers. The pulse program used was a linear ramping progression of 0.1 to 17.33 s with a gradient 6.0 V/cm for 10.5 hrs. Six to seven pairs of gene specific primers were designed for each chitinase genes based on cDNA sequences. They span from 5' end to 3' end. The positions were labeled on (Figure III-1).

III.B.5. Genomic Southern Blotting

Genomic DNA was extracted from erythrocytes by using a high salt method[140]. 10μg DNA was digested for 48h with 120 units of total enzyme, electrophoresed in 0.8% agarose, and transferred onto nitrocellulose membrane by capillary transfer. High-stringency hybridization was performed in 50% formamide/ 6X SSC/ 5X Denhardt's solution / 0.5% SDS/ 100 μg/ml sheared shark plasma DNA at 42 °C for 36-48h. High stringency wash conditions were 2X SSC / 1% SDS at room temperature followed by 0.2

X SSC / 0.1% SDS at 65 °C. Probes used for Southern blot hybridization were generated by PCR from BACs 178-C20, 228-I23, which is set3 probe we used for screening BAC clones (Figure III-1). Probes were labeled with P³².

Table III-1.PCR Primers Used For Screening The BAC Libraries and Clones

BAC Screening Primer	Name	Sequence	Start	End	Product Length
1	Chit01BLSforward	5'-CACAGCTAGGATCCTCCTACATCC-3'	61	84	210
2	Chit01BLSreverse	5'-GGTTCCTGAGAGCCTGGAAGTCTGTC-3'	247	270	210
3	Chit02BLSforward	5'-GCTGATGATGGGATTTGCTACG-3'	825	846	232
4	Chit02BLSreverse	5'-ATTGTCAAATCCTACCCACTGA -3'	1056	1035	232
5	Chit01forwardSet1	5'- TCCTACATCCTATCCTGTTAT -3'	75	95	333
6	Chit01reverseSet1	5'- AGGAACTTGATAACGCTGCT -3'	394	375	333
7	Chit01forwardSet2	5'- CAGCAATGGTGTCCAGTGC -3'	334	352	350
8	Chit01reverseSet2	5'- TCCAGTGTGTGTTCCTCAAG -3'	683	664	350
9	Chit01forwardSet3	5'- GGAACACAACACTGGAGAAAAC -3'	668	689	239
10	Chit01reverseSet3	5'- ACTGTCTGGTGAATGGTCCG -3'	906	887	239
11	Chit01forwardSet4	5'- AACACTGGAGAAAACAGCCC -3'	675	694	379
12	Chit01reverseSet4	5'- TCTTGTCTGGTAACCTTGATG -3'	1053	1031	379
13	Chit01forwardSet5	5'- GACAACATCAAGAGTTACCAGGAC -3'	1026	1049	475
14	Chit01reverseSet5	5'- TACGAGAGGGGGAAGGAAAC -3'	1500	1481	475
15	Chit01forwardSet6	5'- ACCCCACCAATAAGAACAGC -3'	1342	1361	196
16	Chit01reverseSet6	5'- CAACCACAAAACCTTTAGACCC -3'	1537	1516	196
17	Chit02forwardSet1	5'- CGGACATAAATAAGCCCCCTGAC -3'	2	24	360
18	Chit02reverseSet1	5'- GTTGTGTGCCAACTTCCATCC -3'	361	340	360
19	Chit02forwardSet2	5'- TCGGTGGATGGAAGTTTGGC -3'	335	354	400
20	Chit02reverseSet2	5'- TAGAGAGGGCTGTTGTGCTCTGTG -3'	734	711	400
21	Chit02forwardSet3	5'- GAGACCTTCACAGGACACAACAGC -3'	703	726	144
22	Chit02reverseSet3	5'- CGTAGCAAATCCCATCATCAGC -3'	846	825	144
23	Chit02forwardSet4	5'- GCTGATGATGGGATTTGCTACG -3'	825	846	399
24	Chit02reverseSet4	5'- AGGGGAGGTCTGTTCAATGTTG -3'	1223	1203	399
25	Chit02forwardSet5	5'- AATCTGGACCTGGATGACTTTGC -3'	1123	1145	218
26	Chit02reverseSet5	5'- GGGAATCTGCTTTGACATACAGC -3'	1340	1317	218
27	Chit02forwardSet6	5'- GCTGTATGTCAAAGCAGATTCCCC -3'	1317	1340	187
28	Chit02reverseSet6	5'- TTTAGGACGAGCCGAGGTCATC -3'	1503	1482	187
29	Chit02forwardSet7	5'- AAATGATGACCTCGGCTCGTC -3'	1478	1498	415
30	Chit02reverseSet7	5'- TCAACAACTGGGTGCTTCAGC -3'	1892	1871	415

III.B.7. *in situ* hybridization and karyotyping- in collaboration with Dr. Ruth Phillips

Chromosome *in situ* hybridization was performed as described[141]. The probes used for *in situ* hybridization were plasmid DNA isolated from five BAC clones (48-O1, 77-P9, 113-A18, 178-C20 and 228-I23) using the Qiagen Midi-Preparation kit. Those five clones show highest density in the X-ray film of BAC library screening. BAC clones were labeled with Spectrum Orange (Vysis) or Alexofluor 488 (Molecular Probes) using nick translation kit (Vysis).

III.B.8. BAC Sequencing

For sequencing purpose, target BAC plasmids were sheared and subcloned by using TOPO Shotgun Subcloning Kit (Invitrogen). Briefly, plasmids were sheared through a nebulizer (Figure III-3), fragment between 2Kb~900bp was selected. The selected DNA fragment was treated with T4 DNA and Klenow polymerases to yield blunt end, and dephosphorylated with calf intestinal phosphatase. After dephosphorylation, DNA was ligated with pCR4Blunt-TOPO vector. The ligation was transformed into Electrocomp™ *E. coli*. The transformed *E. coli* was plated onto LB agar plate containing 50 µg/ml ampicillin to grow overnight. The white colonies were picked manually to 96 well microtitre plates containing 2x YT medium. The plates grew overnight in the shaker/incubator at 300rpm/37°C. Plasmids were extracted by using Agencourt's Sprintprep plasmid purification kit (Beckman Coulter Company) and sequenced from both directions. Plasmid preparation and sequencing was done by BASlab personnel of COMB.

III.B.9. Assembly

Sequences from each BAC were assembled into scaffolds using both the Phred/Phrap/Consed assembler and Sequencher (Gene Code Corporation). Scaffolds resulting from the different methods of assembly were consistent with the others. Mate-pair inconsistencies were usually from sequences that were in repeat regions of the scaffolds.

III.B.10. Comparative Genomic Analysis

The analysis for syntenic relationships and conservation profiles was done through the annotation of ECRs (Evolutionary Conserved Regions) in the alignments of genomes. For the identification of conserved non-coding elements by multispecies sequence alignments, we used DCODE ECR browsers (<http://ecrbrowser.dcode.org/>), version February 1, 2008. A genomic interval was annotated as an ECR if it was >100 bp and >70% identity as defined by the number of nucleotide matches in a sliding window;

Chitinase sequences from various species were downloaded from the Ensembl (www.ensembl.org) and UCSC (<http://genome.ucsc.edu/>) genome browsers. Multiple sequence alignments were performed by multi-Lagan and displayed using mVista and Genedoc. Sequence and feature files used to generate the alignments shown in Figure III-13. Putative transcription factor binding sites in the conserved motif were predicted using the Transcription Element Search System (TESS; <http://www.cbil.upenn.edu/tess/index.html>) and Transcription Factor Binding Site (TFBS) search (<http://www.cbrc.jp/research/db/TFSEARCH.html>).

III.C. Results

III.C.1. Selection of target BAC clones

Twenty clones were selected from the arrayed OSU-142 BAC library (3× coverage of the haploid genome) by gene specific screening using the P³² labeled probes for Onmy-CHIT.01 & Onmy-CHIT.02 illustrated in Figure III-1 as a red line. Twenty clones were found to provide a hybridization signal and five clones with the strongest hybridization signals were selected; they are 48-O1, 228-I23, 77-P9, 178-C20, and 113-A18. In order to identify whether each BAC clone contained the entire encoding region for each chitinase, a set of PCR primers covering the coding region for each chitinase (six sets for Onmy-CHIT.01, Figure III-1A and seven sets for Onmy-CHIT.02, Figure III-1B) were screened against the five BAC templates (Figure III-1 Tables). The clones selected for shotgun sequencing showed one clear band in PCR reactions for all PCR pair primers listed in Figure III-1. They are BAC 178-C20 containing Onmy-CHIT.01 target sequences and BAC 228-I23 containing Onmy-CHIT.02 target sequences.

In order to check the genetic stability of the selected BACs, four colonies were randomly picked from each plate and amplified to obtain sufficient quantity of plasmid DNA for restriction digest mapping. Each was digested with three different restriction endonucleases (Eco R1, Not 1 and Sal 1) and run on a low-melting agarose gel. As shown in Figure III-2 the four colonies of 178-C20 have identical digestion patterns indicating 178-C20 is a stable clone with no rearrangement occurring during amplification. Similar data were obtained for 228-I23 (result not shown).

Since the size of both BAC plasmids is above the detectable size limit for regular agarose gel electrophoresis, pulsed field gel electrophoresis (PFGE) was employed. From

the results of PFGE we estimate that BAC 178-C20 is around 82Kb (Figure III-3A) in length and BAC 228-I23 (Figure III-4A) is around 145Kb in length. However, the real size should be slightly bigger than these estimates since the BAC plasmids are supercoiled and move faster than the ladder markers in PFGE. When I blotted both PFGE gels onto a nylon membrane for Southern analysis and probed the blot with DIG-labeled probes (probe location as shown in Figure III-1) I found that BAC 178-C20 hybridizes with Onmy-CHIT.01 probe (Figure III-3B). BAC 178-C20 DNA digested with HindIII and XbaI have two hybridization bands with the Onmy-CHIT.01 probe which indicates that the 178-C20 clone contains more than one copy of Onmy-CHIT.01. Digests of BAC 228-I23 clones showed sole hybridization band with Onmy-CHIT.02's probe and did not hybridize with the Onmy-CHIT.01's probe (Figure III-4B). Hence, Onmy-CHIT.01 & Onmy-CHIT.02 are contained on separate BAC clones with apparently two copies of Onmy-CHIT.01 on one BAC.

III.C.2. Southern Blotting of Genomic DNA

In order to investigate whether the BAC genome organization was identical to the native genomic organization in the rainbow trout genome, Southern blotting of genomic DNA was performed under stringent hybridization and washing conditions. Genomic DNA from four fish were digested with three enzymes (EcoRI, EcoRV, HindIII), blotted and probed with P³² labeled sequences identical to that used for probing the BACs. As shown in Figure III-5A, the genomic DNAs from four fish share the same digestion pattern for Onmy-CHIT.01. Since there are no cutting sites for the three restriction enzymes within the probe, the hybridization results suggest that rainbow trout has more than one copy of Onmy-CHIT.01 gene, similar to the expectation found with 178-C20

clone. On the other hand, as shown in Figure III-5B, all digests of BAC 228-I23 have one hybridization band with Onmy-CHIT.02 probe except for one individual cut with HindIII. The intensity of the two bands argues for allelic polymorphism. All in all, the genomic Southern blotting results indicate that rainbow trout has only one copy of Onmy-CHIT.02 and two copies of Onmy-CHIT.01 in its genome.

III.C.3. Chromosome Fluorescent *in situ* hybridization

Using the five BACs (48-O1, 77-P9, 113-A18, 178-C20 and 228-I23) that were shown to contain the coding sequences for Onmy-CHIT.01 & Onmy-CHIT.02 as probes, fluorescent *in situ* hybridization showed that the two chitinases are contained on the same arm of Chromosome 17 (Figure III-6) of the rainbow trout karyotype.

III.C.4. Shotgun sequence and Assembly of BAC clones 178-C20 and 228-I23

Plasmid DNAs from BAC clones 178-C20 and 228-I23 were extracted, sheared to an average size of 900 to 2000 bp (Figure III-7B), recloned, colonies manually picked and sequenced. A total of 1344 and 1440 random clones were picked for 178-C20 and 228-I23, respectively. Sequences from each clone were assembled into scaffolds using both the Phred/Phrap/Consed assembler and the Sequencher assembly (Gene Code Corporation). Scaffolds resulting from the different methods of assembly were consistent with the others. Mate-pair inconsistencies were usually from sequences that were in repeat regions of the scaffolds (Figure III-8A & B).

The majority of sequence gaps of BAC 178-C20 were filled in using primers designed to the unique sequence flanking gaps. Some primers designed to close these

gaps did not produce any PCR products and sequencing reactions with these primers using the BAC clones as template terminated at the same region or were unreadable due to polymerase slippage. All remaining gaps in the 2 BAC clones were flanked by highly repetitive sequences. BAC 178-C20 was assembled to a 86Kbp and a 7Kbp contig, but they are not connected (Figure III-9A). BAC 228-I23 was assembled into a set of scaffolds with gaps (Figure III-9B).

III.C.5. Repeat Masking

Repeat masking was done through Repeatmasker (<http://www.repeatmasker.org/>) and resulted in the masking of 3.99% of the sequence on BAC 178-C20, and for BAC 228-I23, contigs larger than 4K were masked approximately 0.2%~5%. Since there is no rainbow trout or close species specific repetitive elements database, the masking ratio is very low. Alignment with available rainbow trout EST, manually annotating transcribed transposon sequences increased the repeat/transposon content to 6.66% in BAC 178-C20, 11.8% in BAC 228-I23.

Table III-2.Repeat masking Results of three rainbow trout chitinase genes

BAC 228-I23 Contigs	Total Length	Masking by Repeatmasker	Percentage of masking by repeatmasker	Masking by blast with EST	Total bp by blast	Percentage of masking by blast	Total percentage
62K	62510	2738	4.38	815bp(481~1295) 946bp(13436~14317) 676bp(22360~23069) 751bp(27815~28566) 479bp(37436~37915)	3667	5.87	10.25
23KI	23134	266	1.15	919bp(21993~22912) 1624bp(8622~10246)	3045	13.16	14.31
23KII	23065	483	2.09	785bp(18378~19107) 813bp(19376~19977)	1598	6.93	9.02
18K	17985	220	1.22	857bp(3269~4126) 1627bp(13475~15102)	2367	13.16	14.38

11K	11294	26	0.23	581bp(17~598) 563bp(8900~9463) 585bp(9640~10225)	1999	17.7	17.93
6K	6180	310	5.02	none	0	0	5.02
Average							11.8
178-C20	86321	3447	3.99	862bp(782~1647)(2072~2157)(36373~36520) 820bp(74527~75231)(48519~49167)(1261~1945)(36366~36520) 785bp(36597~37320)(35890~36086)(59904~60050)	2467	2.86	6.85

III.C.6. Gene model predication

In silico gene models were performed on both raw assembled scaffolds and scaffolds after repeat/transposon removal. There was no significant difference in gene model predictions between raw and masked sequences; however, the gene models from the raw scaffolds do contain extra exons derived from transposon ORFs. Gene models were predicted by using GENSCAN (<http://genes.mit.edu/GENSCAN.html>). All the predicted gene models are listed in Tables 3 & 4 and maps of the assembled BACS are presented in Figure III-9A&B. Although sequences were performed using repeat masking before gene modeling, there are still some transposon elements in the sequences. There are two Onmy-CHIT.01 and multiple Zinc finger proteins in 178-C20 based on GENSCAN (Figure III-9A), and one Onmy-CHIT.02 in 228-I23 (Figure III-9B).

Table III-3. Gene model predictions for BAC 178-C20.

Predicated Peptide	Start	End	Exons	Blast Result
--------------------	-------	-----	-------	--------------

1	1657	19694	4	Transposase
2	21263	48220	21	Zinc Finger Protein
3	48348	52317	4	Zinc Finger Protein
4	52814	55113	4	Zinc Finger Protein
5	62425	66349	12	Chitinase
6	66356	68521	4	None
7	75746	79652	12	Chitinase
8	79659	81809	4	None
9	81857	84301	2	None

Table III-4. Gene model predictions for BAC 228-I23.

Predicated Peptide		Start	End	Exons	Blast Result
62K contig	1	17927	1484	7	ReO_6
	2	20003	26586	4	Transposase
	3	31113	26638	6	ReO_6
	4	44645	32143	5	None
	5	47932	44726	3	Sidestep protein
	6	48259	51016	2	Somatostatin receptor type 1
23K I	1	717	20150	12	Chitinase
23K II	1	261	10508	4	None
	2	40	10604	4	None
	3	16323	22841	2	None
18K	1	52	785	2	Transposase
	2	13324	2381	5	Transposase
11K	1	1835	3007	3	Resolvase
	2	3012	4958	3	Replication initiation protein
	3	5075	10068	4	Plasmid partition protein
6K	1	5623	342	4	None

III.C.7. Genomic structure of the rainbow trout chitinase genes

The alignment for both rainbow trout chitinase cDNAs with genomic sequences revealed that both chitinase genes are composed of 12 exons and 11 introns (Table III-5).

Table III-5. Genomic structure of the rainbow trout chitinase genes exon sequences are shown in uppercase and introns in lowercase.

Onmy-CHIT.01

Exon no.	Size (bp)	5' splice donor	3' splice acceptor	Intron no.	Size (bp)
1	25	GCGTTGgtaagt	ctctagGACTGG	I	418
2	30	AGCTAGgtaagt	ccatagGATCCT	II	148
3	202	GAACCAgtgaga	taacagGAACAG	III	381
4	57	TCAGCCgtaagt	acccagGTTTAC	IV	88
5	166	CTGCAGgttaga	atctagGAGCTG	V	254
6	125	TGGATCgtcagt	tctcagCGTGTT	VI	138
7	124	GATGTGgtgagt	tctcagGACTAT	VII	144
8	186	TACGAGgtaatt	cattagATCTGC	VIII	214
9	123	GACAAGgtctgt	ttacagATCGAG	IX	335
10	139	GAGCTGgtgaga	tcccagGTTGTG	X	166
11	263	GCCTAaaaaaac	cactccAATAA	XI	149
12	5				

Onmy-CHIT.02

Exon no.	Size (bp)	5' splice donor	3' splice acceptor	Intron no.	Size (bp)
1	25	TTGCAGgtaaaa	ttgaagGTCTGT	I	86
2	27	AATTGGgtgagt	tctaagGCTCGA	II	245
3	205	AGACAGgtttgc	taacagAAATCC	III	87
4	57	ACAACAgtagaga	ccctagGTTTAC	IV	120
5	166	TGCAAGgtgatg	ttgtagGAGCTC	V	197
6	125	TGCTAAGtaaat	ctttagATACCT	VI	392
7	124	AACACAgtagaga	tgccagGATTTT	VII	127
8	183	TATGAGgtagca	ttccagATCTGT	VIII	88
9	117	ACAAAGgtatgc	cctaagGTCGGC	IX	131
10	133	ACATTGgtaaat	tgccagGGGAAC	X	154
11	191	CCCTAGgactgt	taacacAATAAA	XI	1488
12	6				

III.C.8. Comparison of the two copies of Onmy-CHIT.01 genes

In order to rule out the possibilities that the existence of the two copies of Onmy-CHIT.01 was due to assembly errors, the predicted restriction enzyme cleavages were

examined for the sequence region containing both copies and compared to the southern blot results presented earlier (Figure III-5). This region is around 17 Kbps in length, and was found to have two XbaI cutting sites which are 2648 bp apart, one HindIII cutting site, and no SalI cutting site; both XbaI and HindIII cutting sites reside outside of coding regions of both Onmy-CHIT.01 copies. This predicted map agrees very well with southern blotting results. As shown in Figure III-5, two bands are observed in the XbaI and HindIII digests, while only one band is seen in SalI digest. The size of the lower band in XbaI is smaller than in HindIII, which is due to 2648 bp between two XbaI cutting sites. All these data illustrate that 178-C20 contains two copies of Onmy-CHIT.01 and that the Contig assembled is legitimate reconstruction of the native genomic sequence.

The two copies of Onmy-CHIT.01 were carefully compared. As shown in Table III-6, the coding range for these two copies are almost identical with the transcribed cDNAs only have one base pair difference. However, the 5' sequences are quite different which could indicate that these two copies are regulated differently.

Table III-6. Identities between two Onmy-CHIT.01 copies.

Size	First Copy	Second Copy	Identity
Upstream of ATG	2000	2000	44.60%
Upstream of ATG	1000	1000	50.10%
Upstream of ATG	500	500	61.60%
Upstream of ATG	200	200	94.30%
Coding Range	3863	3859	94.40%
Transcribed cDNA	1446	1446	99.90%

Both Onmy-CHIT.01 & Onmy-CHIT.02 genomic structures are surprisingly conserved when compared with their mammalian equivalents (Table III-6, Figure III-10A & B). The length difference of the 9 exons for the chitinase genes in the three species compared is less than 6 base pairs. However, the intron length is much more variable (Table III-7).

Table III-7. Comparison of Genomic structure on chitinases genes. Yellow cell indicate length difference among three genes is less than 6bps.

No	Onmy-CHIT.01		Human AMCase		Mouse AMCase	
	Exon	Intron	Exon	Intron	Exon	Intron
			35	19451		
1	25	418	93	1198	28	1237
2	30	148	30	473	30	494
3	202	381	202	2150	202	5994
4	57	88	57	675	57	673
5	166	254	166	2247	166	5620
6	125	138	125	182	125	178
7	124	144	124	385	124	262
8	186	214	186	443	186	1600
9	123	335	120	89	120	88
10	139	166	142	746	142	812
11	263	149	345		346	
12	5					
	1445	2435	1625	28039	1526	16958
Gene Size	3880		29664		18484	
No	Onmy-CHIT.02		Human Chitotriosidase		Mouse Chitotriosidase	
	Exon	Intron	Exon	Intron	Exon	Intron
					121	22623
1	25	86	37	1051	132	1489
2	27	245	30	2664	30	2952
3	205	87	202	566	202	571
4	57	120	56	1390	57	1186
5	166	197	166	237	166	175
6	125	392	125	811	125	854
7	124	127	124	2354	124	1652
8	183	88	186	336	186	856
9	117	131	114	1352	108	1003
10	133	154	127	607	127	500
11	191	1488	467		333	
12	6					
	1359	3115	1634	11368	1711	33861
Gene Size	4474		13002		35572	

III.C.9. Promoter region prediction

Putative transcription initiation sites were predicated by Promoter 2.0 Prediction

Server (<http://www.cbs.dtu.dk/services/promoter/>) and Neural Network Promoter

Prediction (NNPP) (http://www.fruitfly.org/seq_tools/promoter.html). These two

softwares use different algorithms and provide different prediction results. Promoter 2.0 uses a modified neural network and genetic algorithm method to recognize a set of discrete subpatterns, with variable separation, as one pattern[142]. NNPP combines recognition of the TATA box and the Initiator region (Inr), using the time delay neural net architecture, which allows for variable spacing between the features[143]. Both of these prediction softwares have limitations. In a review authors evaluated several promoter prediction tools by comparing prediction results with experimental results[143]. NNPP has the highest sensitivity (number of promoters correctly detected), but also with the highest specificity (number of false positives), Promoter 1.0 (previous versions of promoter 2.0) has second to highest sensitivity with second to highest specificity in nine tools authors considered.

The predictions from NNPP are more consistent with my 5'RACE results. The two copies of CHIT.01 have very high identity (97%) within 185 bp upstream of the ATG start codon, thus, I used the second copy as my template to analyze promoter region. 5' RACE results show there is 12 bp 5'UTR, and a TATA box fragment is found 31 bp upstream 5'UTR. NNPP's results are identical to this finding. Promoter 2.0's results indicate a regulatory region around 600 bp upstream of the ATG.

In the case of Onmy- Chit02, 5'RACE showed a 48 bp 5'UTR, and I am unable to detect a TATA box fragment within 100 bp upstream 5'UTR. The NNPP predictions shows that Transcription Starting Site (TSS) is 10 bp upstream ATG with no TATA box. Promoter 2.0 predicted TSS is around 500 bp downstream of the ATG.

III.C.10. Transcription Factor Binding Site Prediction

I searched for regulatory element binding sites 2000 bps upstream of the ATG in the two Onmy-CHIT.01 copies and Onmy-CHIT.02 gene using the TFsearch engine Version 1.3 (<http://www.cbrc.jp/research/db/TFSEARCH.html>). The threshold was set to 85.0. The list of all putative transcription factors found is summarized in Table III-8. There are a total of 225 predicted transcription factors binding sites for Onmy-CHIT.01 first copy, 192 for Onmy-CHIT.01 second copy, and 171 for Onmy-CHIT.02. Comparing the genes, five factors are unique to first copy, two to second copy, and seven to Onmy-CHIT.02. An especially unique transcription factor binding site in the first copy of Onmy-CHIT.01 was the nuclear factor kappaB (NFκB) binding site.

Table III-8. Comparison of transcription factor binding sites of three rainbow trout chitinase genes. Grey cell indicates unique transcription factor binding site of the gene compared to the other two genes. Red letter indicates the transcription factor binding site associated with chitinase expression.

TF	Count for Copy one	TF	Count for Copy two	TF	Count for Chit02
AML-1a	5	AML-1a	5	AML-1a	5
AP-1	9	AP-1	4	AP-1	2
AP-4	1				
C/EBP	5	C/EBP	3	C/EBP	2
		C/EBPa	1	C/EBPa	2
C/EBPb	1	C/EBPb	3	C/EBPb	3
CDP	3	CDP	2		
CdxA	48	CdxA	53	CdxA	48
c-Ets-1	3				
Clox	1				
CP2	1	CP2	2	CP2	1
				CREB	2
CRE-BP	1			CRE-BP	2
deltaE	4	deltaE	1	deltaE	4
					2
E2F	1			E2F	2
				E47	1
		E4BP4	1	E4BP4	1
Evi-1	3	Evi-1	6		

GATA-1	31	GATA-1	19	GATA-1	12
GATA-2	17	GATA-2	13	GATA-2	7
GATA-3	7	GATA-3	7	GATA-3	4
GATA-X	4	GATA-X	1	GATA-X	2
				Gfi-1	1
		HFH-2	1	HFH-2	1
				HNF-1	1
HNF-3b	3	HNF-3b	1	HNF-3b	2
HSF1	2	HSF1	2	HSF1	1
HSF2	4	HSF2	3	HSF2	2
Ik-2	3	Ik-2	1	Ik-2	3
				IRF-2	1
		Lyf-1	1	Lyf-1	4
MyoD	1	MyoD	1		
MZF1	4	MZF1	8	MZF1	4
		NF-E2	1		
NF-kapaB	1				
Nkx-2.	7	Nkx-2.	6	Nkx-2.	6
p300	1	p300	3		
Pbx-1	4	Pbx-1	2	Pbx-1	1
		Pbx1b	1		
RORalp	3	RORalp	4	RORalp	2
S8	6	S8	3	S8	2
Sox-5	4	Sox-5	4	Sox-5	1
Sp1	1				
SREBP-	1			SREBP-	2
SRY	20	SRY	11	SRY	16
STATx	2	STATx	2	STATx	2
TATA	6	TATA	4	TATA	1
Tst-1	1	Tst-1	2	Tst-1	1
USF	1	USF	2	USF	1
v-Myb	2	v-Myb	1	v-Myb	3
		XFD-1	1	XFD-1	1
				XFD-3	1
				YY1	1
1-OCT	3	1-OCT	6	1-OCT	10
Grand Total	225	Grand Total	192	Grand Total	171

III.C.11. Comparative Genomic Analysis

To identify candidate chitinase regulatory elements, I compared the genomic loci of 3 mammalian species: human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), with four teleost species - the rainbow trout, pufferfish (*Fugu rubripes*), tetraodon (*Tetraodon nigroviridis*) and zebrafish (*Danio rerio*). Multi-species sequence

comparisons were performed with Lagan and an overview of the alignment is displayed in Figure III-11 as a Vista plot using the Onmy-CHIT.01 first copy as reference. High levels of sequence conservation are observed across the coding region among examined sequences. However, there is little to no conservation detected in any regulatory region of the other vertebrates. There appears to be some conservation in the 5' UTR of the two Onmy-CHIT.01 genes but none with the Onmy-CHIT.02 gene from the same species.

As shown in Figure III-12, the evolutionary conserved regions (ECR) for the two human chitinase genes were compared with the Fugu, Tetraodon, zebrafish, frog, chicken, rhesus macaque, chimpanzee, opossum, dog, cow, mouse, and rat genomes. The ECR browser. (<http://ecrbrowser.dcode.org/>) was used. The twelve genomes that were compared to the human region are plotted as horizontal layers of conservation diagrams and the small image icon at the right side of the plot represents the species corresponding to the alignment. However, there is no ECR for either human chitinase gene outside what is found in other mammals. Perhaps the comparison to mammals is uninformative over great evolutionary distance.

I therefore restricted the analysis to fish only. The Onmy-CHIT.02 gene was aligned with the zebrafish genome that was used as base genome for the ECR browsers. The result is shown in Fig III-13. Three zebrafish genes were found; their mRNA Genbank accession numbers are NM_213050, NM_213249, and NM_213213, and their genomic accession number is NC_007122. A total of 19 ECRs were found when aligned with the Fugu genome, and 11 with opossum, but no ECRs with other species. Among the 19 ECRs with Fugu, 3 are located in the intergenic region since they are less than 2000bp away from the next exon. The detailed three ECRs and their conserved transcription

factor binding sites are presented in Figure III-14. I also did the same analysis on the Onmy-CHIT.01 gene; however, there are no detectable ECRs when aligned with any existing fish genomes.

III.D. Discussion

I have determined that the OSU clonal line of the rainbow trout (*Onchorhynchus mykiss*) possesses at least two chitinase genes (designated Onmy-CHIT.01 and Onmy-CHIT.02). Onmy-CHIT.01 is expressed primarily in gastric tissue while Onmy-CHIT.02 is expressed in the myleoid lineage cells of immune related organs, such as the spleen, liver, etc. These two genes share 58% amino acid identity in their coding regions and the predicted proteins possess all the characteristic protein motifs found in the Family 18 glycosyl hydrolyases (E.C 3.2.1.14). Both Onmy-CHIT.01 and Onmy-CHIT.02 genes are located on chromosome 17 based on fluorescent *in situ* hybridizations. The genomic arrangement of these chitinase genes based on complete sequencing of BAC library clones found two adjacent copies (9kb apart) of Onmy-CHIT.01 with transposons and transposases elements nearby. The two copies are similar in coding region with only the first intron being different. Both contain 12 exons, spanning around 4 Kb in the genome. I detect only one copy of Onmy-CHIT.02 in the rainbow trout genome, which also contains 12 exons, and spans 5 kb in length. The promoter sequence of Onmy-CHIT.02 is highly divergent with both copies of Onmy-CHIT.01 and may explain the tissue expression differences seen for these two homologs. Southern blot analysis results of genomic DNA are consistent with the BAC clone gene arrangements. Onmy-CHIT.02 is the first sequenced non-mammalian chitinase gene that has been shown to be expressed in macrophages and neutrophils.

As discussed earlier, chitinase amino acid sequences are relatively well conserved within the vertebrates. My data indicates that this extends to their gene structure.

Chitinases genes from rainbow trout, mouse and human all have 11~12 exons, the length

difference of 9 exons of three species chitinase genes are less than 6 base pairs. But length of 5' and 3' ends are not comparable. Likewise, intron length is not conserved among vertebrates, especially for intron 1 in human AMCase which is 19Kb in length. Similarly, intron 1 of mouse chitotriosidase is 22Kb, and in both cases, translation start sites are located in the second intron. It has been shown that first introns enhance gene expression more than any others introns in both mouse[144]and human[145], and first intron evolves faster than other introns[146]. Moreover, first introns tend to be longer than introns in other positions of the gene[147]. Due to the significance of first introns, I did an alignment analysis of these two long introns with the 5' UTRs but found no conserved regions.

III.D.1. Transcriptional Control of Vertebrate Chitinases

Within the unique transcription factors of Onmy-CHIT.02, interferon regulatory factors-2 (IRF-2) is very well studied in mammalian systemd and is likely to be associate with secretion of chitinase. Besides NFκB and IRF-2, there are four unique transcription factors of the first Copy of Onmy-CHIT.01, two of the second copy of Onmy-CHIT.01and six of Onmy-CHIT.02. They are AP-4, c-Ets-1, Clox, Sp1; NF-E2 and Pbx1b; CREB, E47, Gfi-1, HNF-1, XFD-3, and YY1(Table III-9). Currently, no reports associated these factors with chitinase expression; however, some of them are global transcription factors, which means they may indirectly affect chitinase expression.

In human, IFN-γ, TNF-α and LPS promote chitotriosidase gene expression in macrophages[148]. Since it is known that macrophages treated with IFN-γ & TNF-α develop an increased cytotoxic activity against intracellular microorganisms and tumor

cells, it is very likely that chitotriosidase is involved in a cellular response elicited by this regulatory cytokine[148].

In rainbow trout, both IRF-1 and IRF-2 has been cloned, and IRF-2 represses the expression of IFN- γ , which could perform as a negative regulator of Onmy-CHIT.02[149].

The transcription factor NF κ B is activated by numerous stimuli. Once NF κ B is fully activated, it participates in the regulation of various target genes in different cells to exert its biological functions. Recent studies have shown that NF κ B may function more generally as a central regulator of stress responses, since different stressful conditions, including physical stress, oxidative stress, and exposure to certain chemicals, also lead to NF κ B activation. There are 5 different NF κ B family members (p65, c-Rel, RelB, p50 and p52), which all bind to a similar consensus sequence and can be activated by several pathways that converge on I κ K[150]. NF κ B is associated with the expression of stomach specific genes, such as gastrin which induces expression of CXC chemokines through activation of NF κ B in gastric epithelial cells that express the gastrin receptor[151].

Data showing chitinase induction with NF κ B is not very well validated. However, it is known that induction and continued secretion of chitinase 3-like protein 1 (Chi3L1) in articular chondrocytes requires sustained activation of NF κ B[10]. In addition, persistent activation of nuclear factor-kappaB has been associated with the development of asthma. The inhibition of one of the inhibitors of NF κ B markedly suppressed the mRNA expression of acidic mammalian chitinase, Ym1, and Ym2 in mice[11].

In rainbow trout, cortisol directly up-regulated the transcriptional response of NF κ B in macrophages [152]. Only the first copy of Onmy-CHIT.01 has a NF κ B binding site,

which could very well indicate that this copy is under regulation under stress conditions, but the other copy may constitutively expressed.

While I found a TATA element in Onmy-CHIT.01 I found no TATA box in Onmy-CHIT.02. This is not surprising given that recent research has showed that only 10~20% of mammalian promoters contained a functional TATA box, and that TATA box containing promoters are usually associated with tissue or context-specific genes. Similar data to this phenomena are also found in *Drosophila melanogaster* and *Arabidopsis thaliana* [153].

Table III-9. List of Transcription Factors that are possibly involved in chitinase gene regulation.

Abbreviation	Name of transcription factors	Comments
AP-4	activator protein 4	AP-4 performs as either an enhancer or a repressor of many genes[154, 155]. AP-4 also interact with other transcription factors by overlapping binding sites of the recognition sequences of other transcription factors such as AP-1/Jun[154].
c-Ets-1	the cellular homologue to the viral ets (<i>v-ets</i>) gene	The name "Ets" stems from a sequence that was detected in an avian erythroblastosis virus, E26, where it formed a transforming gene together with gag and <i>c-myb</i> . This sequence was called E26 transformation specific sequence or Ets[156]. c-Ets-1 plays a distinct roles in haematopoietic cell differentiation and participates in the regulation of invasive behavior of many normal and tumor cells [156].
Clox	Cut-like homeo box	Clox is a transcription factor specific for developmentally important genes in a broad variety of organisms, from <i>Drosophila</i> to mammals[157]. A common feature of the <i>cut</i> homeo proteins is the presence of three evolutionarily conserved <i>cut</i> repeats of unknown function[158]. The SP1 transcription factor contains a zinc finger protein motif, by which it binds directly to DNA and enhances gene transcription.

		Sp1 involveds in gene expression in the early development of an organism. It contains a zinc finger protein motif, by which it binds directly to DNA and enhances gene transcription[159].
NF-E2	nuclear factor erythroid 2	NF-E2 is crucial for regulation of erythroid-specific gene expression[160].
Pbx1b	pre-B-cell leukemia transcription factor 1b	Pbx1b is a homeodomain protein that functions in complexes with other homeodomain-containing proteins to regulate gene expression during embryogenesis and oncogenesis[161].
CREB	cAMP response element-binding protein	<p>CREB is a stimulus-induced transcription factor that responds rapidly to phosphorylation and/or coactivator activation. It dimerizes with itself or other family members and bind to the cyclic AMP response element (CRE) on target genes. CREB controls the expression of a number of genes, including other transcription factors[162].</p> <p>E47 along with E12 are encoded by one gene, designated E2A, and arise through alternative splicing of the exon encoding the helix-loop-helix (HLH) domain 56. E12 and E47 bind to sequence motifs, termed E-boxes that are found in the regulatory regions of numerous lineage-specific genes[163]. They are key regulators of both B and T lymphocyte differentiation[164].</p>
Gfi-1	growth factor independence 1	Gfi-1 encodes a zinc finger transcription factor whose expression is important for interleukin-2 signaling[165].
HNF-1	hepatocyte nuclear factor 1	HNF-1 is a liver specific transcription factor family, which encodes both HNF1 α and HNF1 β . This family is very conserved through vertebrae[166].
XFD-3	Xenopus fork head domain factor	XFD-3 is a <i>Xenopus laevis</i> gene which encodes the homologs to mammalian HNF-3 β [167], HNF-3 is a family including three distinct hepatocyte transcription factors, HNF-3 α , -3 β , -3 γ . They expressed in the lung and are essential participants in liver- and lung-specific gene transcription[168].
YY-1	Yin-Yang 1	YY-1 binds to the promoter regions of several T-cell cytokine genes, such as IL-2 & IL-4[169].

III.D.2. Chitinase Orthology and Synteny

Both mouse and human chitinase genes are located on syntenic chromosomes (Figure III-15 & 16). AMCase's synteny contains AMCase, Chitinase 3-like 2, Oviductal glycoprotein 1, ATP synthase, and Adenosine A3 receptor. These five genes span 230Kbp in the mouse chromosome 3F3 region and 220Kbp on human chromosome 1P13 region. In both human and mouse, not too far from Chitotriosidase (CHIT1), there is glycoprotein called Chitinase 3-like 1 (CHI3L1), also known as YKL40 or HC-gp39, which shares structure similarity to chitinase but without chitinolytic activity. It is located between 1q31 to 1q32 on Chromosome 1. This chitinase like protein is assumed to be derived by duplication of chitotriosidase with subsequent lost of important catalytic residues.

Except for chitinase no other syntenic genes could be found in the two rainbow trout BAC clones. Both of rainbow trout chitinase genes are located on the short arm of chromosome 17 as shown in Figure III-11, which is more physically close to each other than mouse and human chitinases.

Table III-10. Genomic location of two chitinases in different species.

Species	Chitotriosidase (CHIT1)	AMCase (CHIT3, acidic, CHIA)
Human	1q32	1p13
Mouse	1E+04	3F3
Rainbow Trout	17 short arm	17 short arm

The lack of synteny could be due: 1) sequenced region is very limited, less than the spanning length of a synteny; 2) rainbow trout chromosomes are not as concise as mammalian chromosomes, which contains far more non-coding region; 3) rainbow trout

because of its tetraploidization and subsequent recombination have scrambled chromosome synteny.

III.D.3. Gene Copy Variation of Vertebrate Chitinases

Understanding the historical relationships and the molecular mechanisms underlying the evolution of gene families is a challenging goal in the study of evolution. The origin of genome complexity in metazoan is generally thought to be linked to gene duplication events. In this study, three rainbow trout chitinase genes were found and sequenced in the rainbow trout genome. Two genes are duplicated copies of Onmy-CHIT.01 that may relate to the high expression levels observed in the stomach. It is common phenomena in vertebrates to have duplicated genes; approximately 5–10% of the human genome consists of low-copy repeats[170]. Copy numbers of a particular gene can vary among different species. For example, the number of genes encoding lysozyme varies greatly among mammals, with humans and pigs carrying 1 gene, mice and camels 2, and cows and sheep 10[171].

In ruminants, within ten copies lysozyme genes, several are expressed in the gastrointestinal tract. One copy is expressed in innate immunity cells. Copy number polymorphisms are not a common feature of innate immune genes[172], but are fairly common in aspartic proteinases, which make up most of digestive enzyme of stomach[173]. Gene duplication is considered an important evolutionary mechanism leading to new gene functions. One gene copy arising from gene duplication retains the ancestral function, whilst the other becomes subject to directional selection for some novel functions. This birth and death model could explain the case of AMCase. According to this model, long-term persistence of two paralogous genes is possible only

with the acquisition of functional innovation. There is a paralogous gene-CHI3L2 locating next to AMCase gene in both human and mouse genome (Figure III-14). It is likely that CHI3L2 was a derivative of AMCase, which maintains the protein structure of chitinase but does not have catalytic activity. However, it gains a new role in articular chondrocytes[174].

III.D.4. Evolutionary Origin of Vertebrate Chitinases

According to the data presented, the evolution of chitinase is marked by numerous gene duplication events leading to the segregation of various groups of paralogs (Figure III-17). Clearly, additional data are required to clarify the fate of the chitinase gene during vertebrate evolution. Since only known chitinase genes were used to do the alignment, many unknown genomic regions are left behind. In order to compensate for this bias I did a full genome search. A full-length sequences plus 2000bp up and down stream of both human chitinase genes was used as the inquiry for the ECR browser. (<http://ecrbrowser.dcode.org/>) As shown in Figure III-15, the conservation profiles of the human region in comparison with the fugu, tetraodon, zebrafish, frog, chicken, Rhesus Macaque, chimpanzee, opossum, dog, cow, mouse, and rat genomes are shown. The twelve genomes that were compared to the human region are plotted as horizontal layers of conservation diagrams and the small image icon at the right side of the plot represents the species corresponding to the alignment. However, there is no ECR existing in this region outside of mammals in both human genes. Since my goal is to explore the ECRs across the vertebrates, especially among lower vertebrates, I did not do further analysis on ECRs of mammalian chitinase gene.

Furthermore, no conserved region can be detected in regulatory parts of chitinase genes across the vertebrates. This could indicate that regulation of chitinase varies greatly between the species. This matches with the experimental evidence that there are big differences on mRNA tissue distribution between human chitotriosidase and mouse chitotriosidase.

But I did find the ECRs in zebrafish and fugu potential chitinase gene regulatory regions when aligned with Onmy-CHIT.02. This suggests that chitinase genes follow certain regulation pattern within the teleost fishes. But the confirmation of regulatory elements needs additional sequences from fishes and further experimental evidences.

Figure III-1. Location of primer sets used for screening the rainbow trout genomic BAC clones based on Chitinase cDNA sequences.

A. Onmy-CHIT.01 cDNA

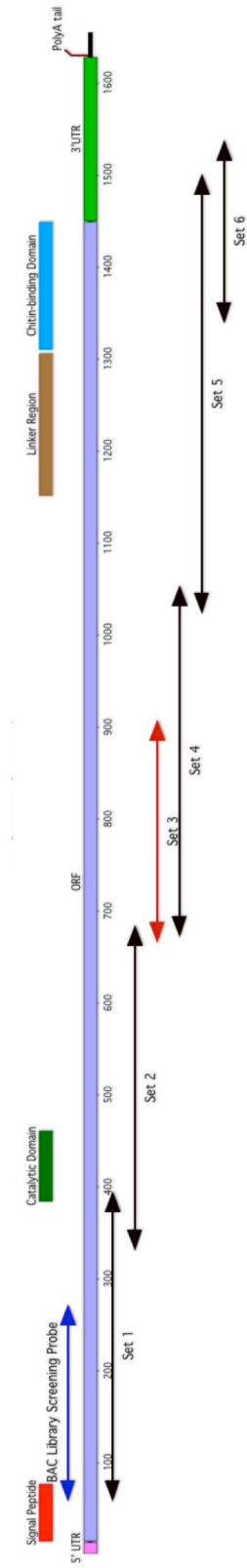
B. Onmy-Chit.02 cDNA.

Six primer sets (Table III-1) were used for Onmy-Chit.01, seven for Chit.02 (Table III-1).

The location of important structural motifs are mapped on the drawing for orientation.

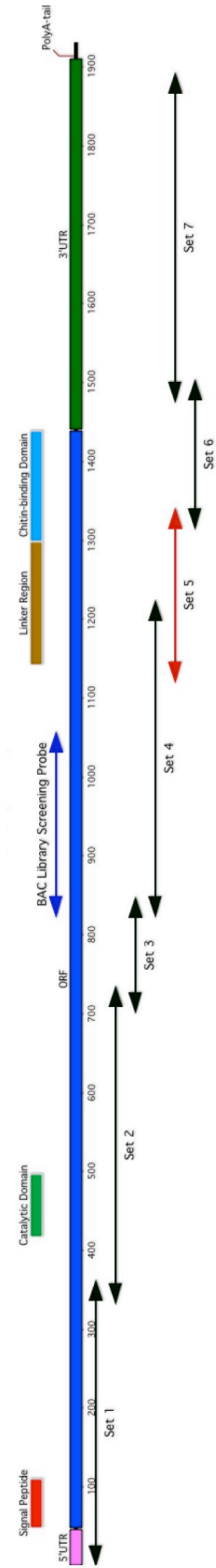
The red primer sets were used to generate probes (P^{32} and Dig-labeled) for southern blotting. The blue primer sets were used to generate probes (P^{32}) for screening BAC library. The Tables underneath each drawing indicate the predicted amplicon lengths based on the cDNA sequence and the observed amplicon length when amplifying rainbow trout genomic DNA. The amplicons were amplified using different templates including the cDNA, genomic DNA and BACs from the five clones. +++ indicates strong amplification, ++ indicates weak but still detectable amplification and empty cells indicate no observed amplicon. Bold light blue highlights the clone selected for shotgun sequencing.

A



Onmy-Chit.01	Template	set1	set2	set3	set4	set5	set6
Amplicon Size	cDNA	333	349	239	379	475	196
Approximate Amplicon Size	Genomic DNA	1000	800	500	850	1200	200
1	48-O1	+++					
2	77-P9						
3	113-A18	++		++	++		
4	178-C20	+++	+++	+++	+++	+++	+++
5	228-I23						

B



Onmy-Chit.02	Template	set1	set2	set3	set4	set5	set6	set7
Amplicon Size	cDNA	360	400	144	399	218	187	415
Approximate Amplicon Size	Genome DNA	800	1200	200	600	350	1750	500
1	48-O1	++						
2	77-P9	+++	+++	+++	++	+++	+++	+++
3	113-A18	++						
4	178-C20	+++	++	+++		++		++
5	228-123	+++	+++	+++	+++	+++	+++	+++

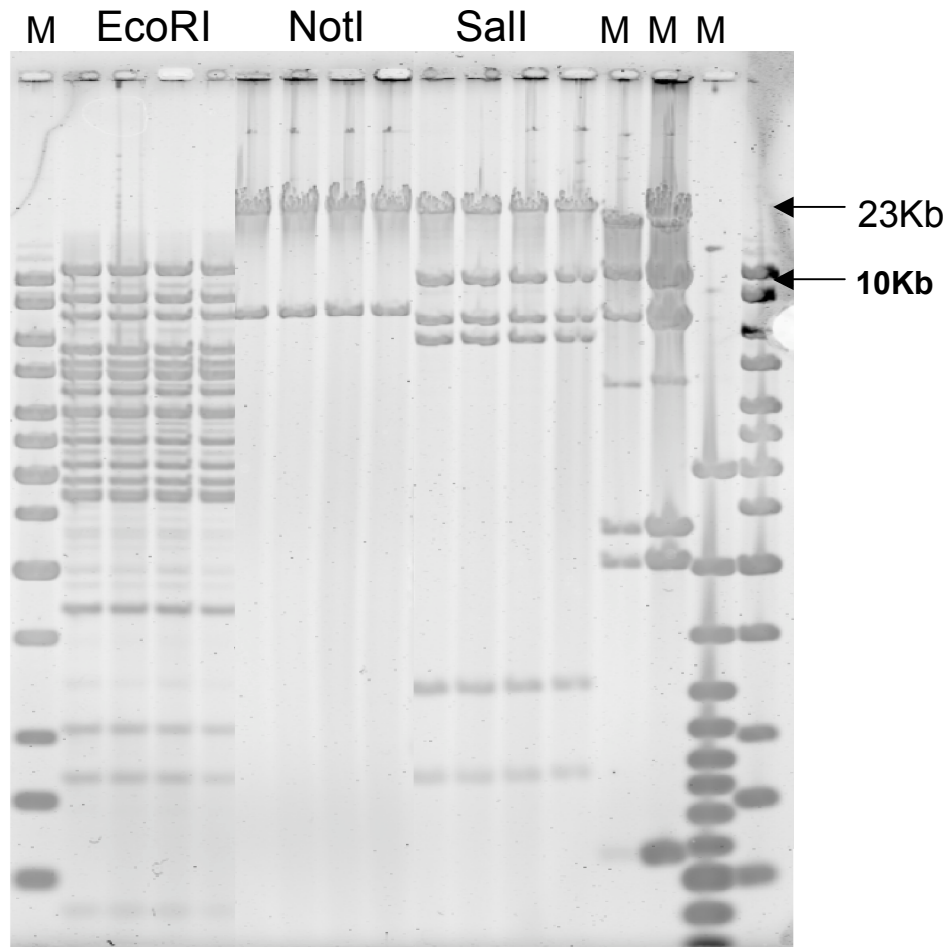
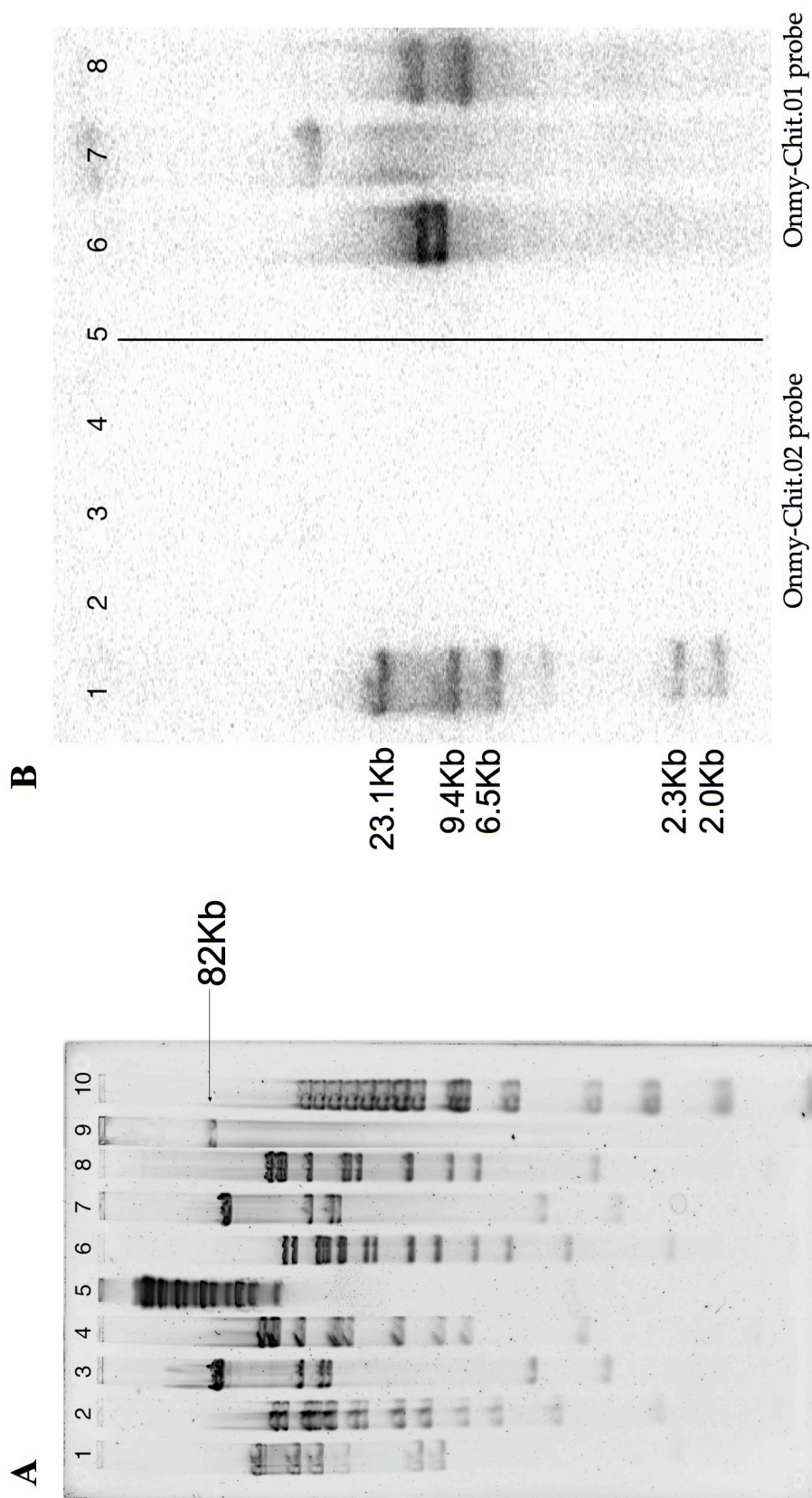


Figure III-2. BAC clone stability test. Plasmids from four BAC clone preparations of 178-C20 were digested with three restriction endonucleases (EcoRI, NotI & Sall) separately. The digests were run on 1% Seaplaque GTG agarose (Cambrex) overnight at the manufacturer recommended conditions. Five DNA ladders were used, M1&M5 were GeneRuler 1Kb DNA ladder (Fermentas), the top band indicates 10Kbp; M2&M3 were λ DNA/Hind III Fragments (Invitrogen), the top band indicates 23Kbp; M4 was GeneRuler 100bp DNA ladder Plus (Fermentas), the top band indicates 3000bp.

Figure III-3. Pulse field gel electrophoresis (PFGE) on restriction enzyme digests of BAC 178-C20.

- A. BAC 178-C20 restriction enzyme digestion pattern. Lane 1, DIG-labeled λ PFGE marker; lane 2, HindIII; lane 3, Sall; lane 4, XbaI; lane 5, middle range PFG marker (New England Biolab); lane 6, HindIII; lane 7, Sall; lane 8, XbaI; lane 9, full plasmid; lane 10, 1Kb ladder.
- B. Southern blot of the PFGE gel hybridized with probes for Onmy-Chit.02 & Onmy-ChitChitChit.01 Left half was hybridized with Onmy-Chit.02 specific probe; right half was hybridized with Onmy-Chit.01 specific probe. Only the Onmy-Chit.01 probed showed hybridization.

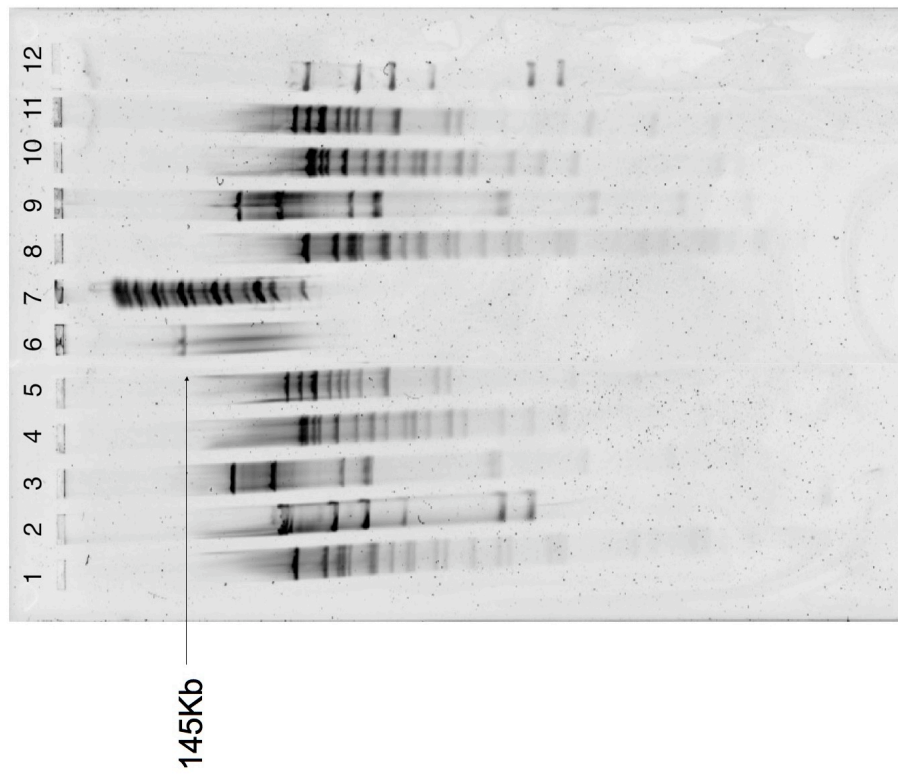


BAC 178-C20

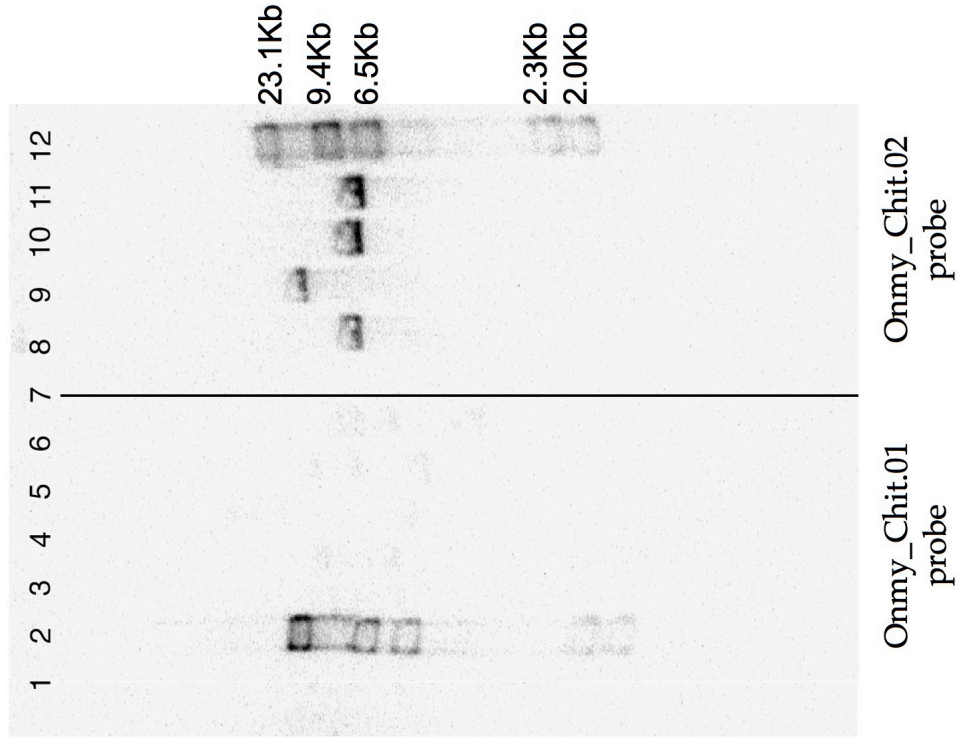
Figure III-4. Pulse field gel electrophoresis (PFGE) on restriction enzyme digests of BAC 228-I23 plasmids.

- A. BAC 228-I23 restriction enzyme digestion pattern. Lane 1, HindIII; lane 2, DIG-labeled λ PFGE marker; lane 3, SalI; lane 4, XbaI; lane 5, KpnI; lane 6, full plasmid; lane 7, middle range PFG marker (New England Biolab); lane 8, HindIII; lane 9, SalI; lane 10, XbaI; lane 11, KpnI; lane 12, DIG-labeled λ PFGE marker.
- B. Southern blot of PFGE gel. Left half was hybridized with Onmy-Chit.01 specific probe; right half was hybridized with Onmy-Chit.02 specific probe. Only the Onmy-Chit.02 probed showed hybridization.

A



B



BAC 228-I23

Figure III-5. Southern blot of restriction enzyme digested rainbow trout genomic DNA. DNA from four individual fish was used. Probes used for hybridizing with southern membrane were illustrated in Figure III-1. 10µg DNA was digested with HindIII, EcoRI and EcoRV for 48h with 120 units of total enzyme. The digests was electrophoresed in 0.8% agarose, transfer to a nitrocellulose membrane, washed twice conditions with 2X SSC / 1% SDS at room temperature followed by washing with 0.2 X SSC / 0.1% SDS at 65 °C.

- A. Hybridized with Onmy-Chit.01 specific probe
- B. Hybridized with Onmy-Chit.02 specific probe

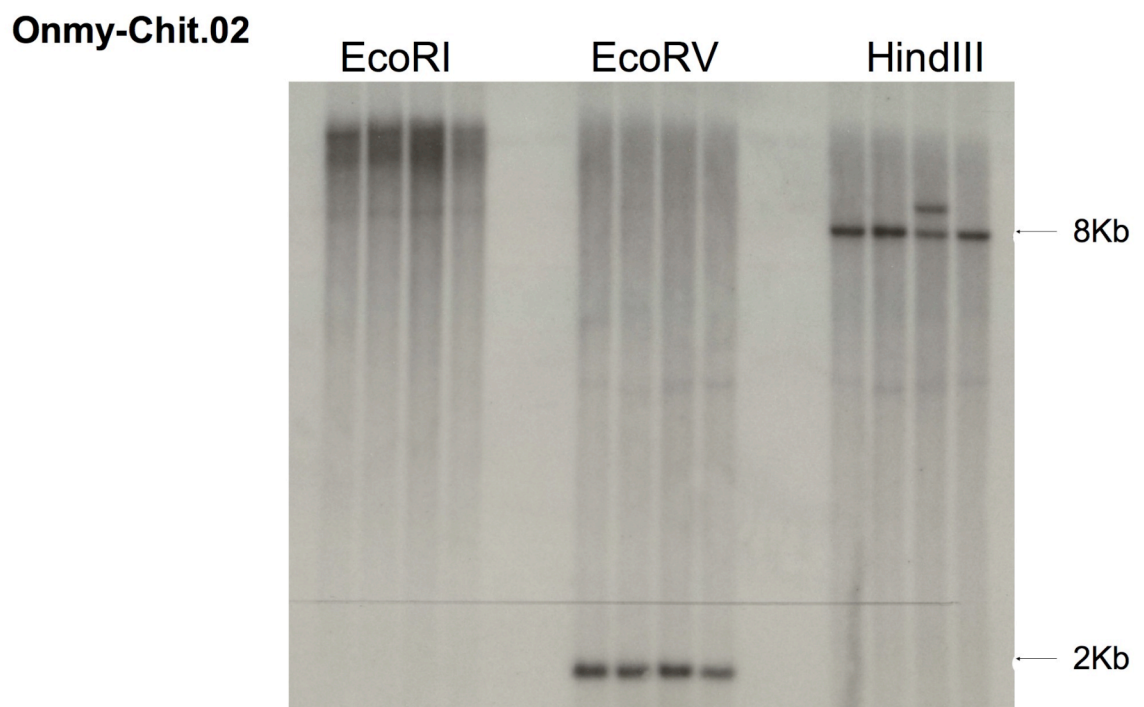
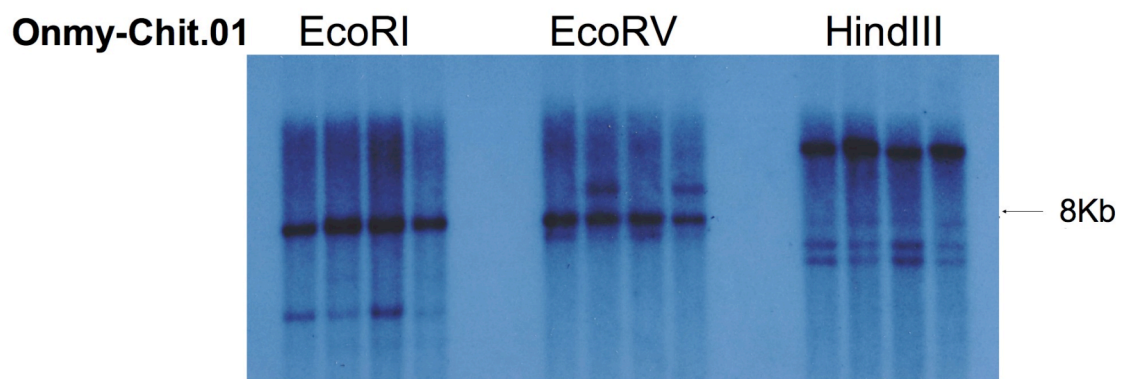
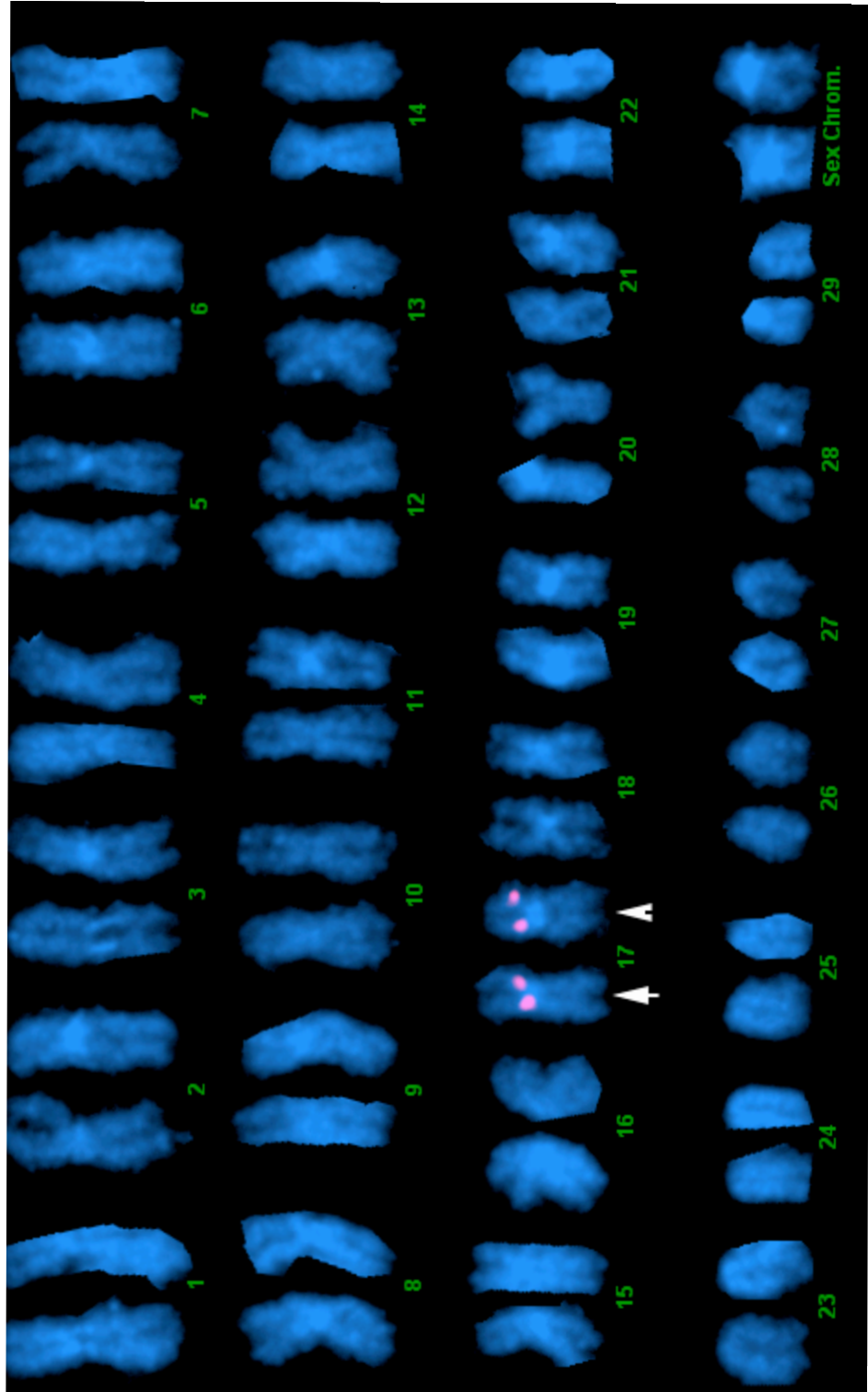


Figure III-6. Fluorescence *in situ* hybridization analysis on chromosome spreads for rainbow trout showing hybridization of Onmy-Chit.01&02 containing BACs (48-O1, 77-P9, 113-A18, 178-C20 and 228-I23) only to Chromosome 17.



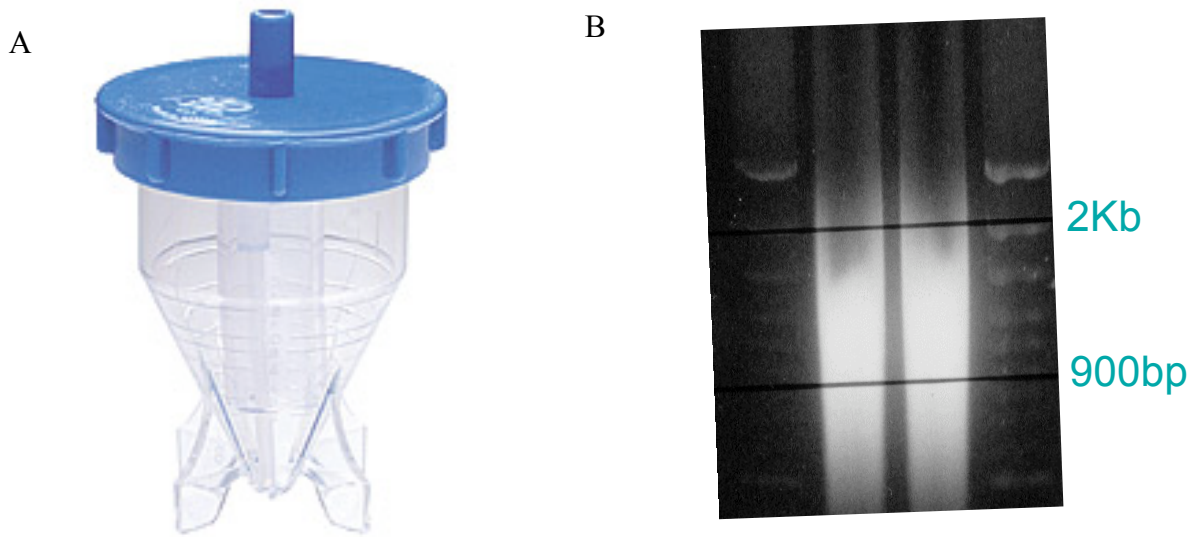


Figure III-7. Random shearing and shotgun sequencing of BAC clones.

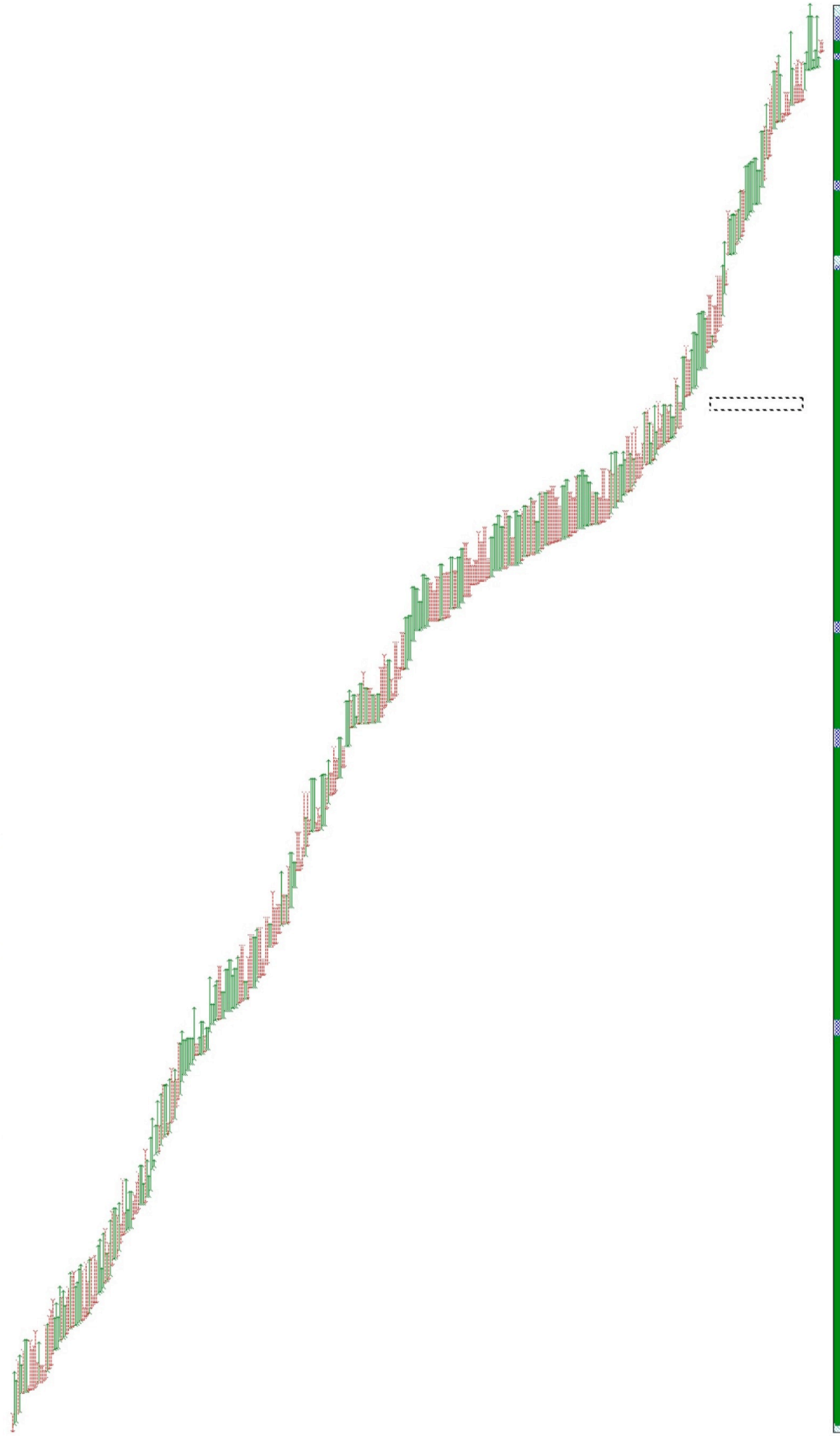
A. Nebulizer used to randomly shear the BAC plasmid DNA

B. DNA fragments between 2000~900bp from the sheared DNA were selected for subcloning into pCR4Blunt-TOPO vector. After transfection, clones were picked manually from LB plates to 96 well plates for plasmid extraction. For BAC clone 178-C20, 14 plates were prepared; for BAC clone 228-I23, 15 plates were prepared.

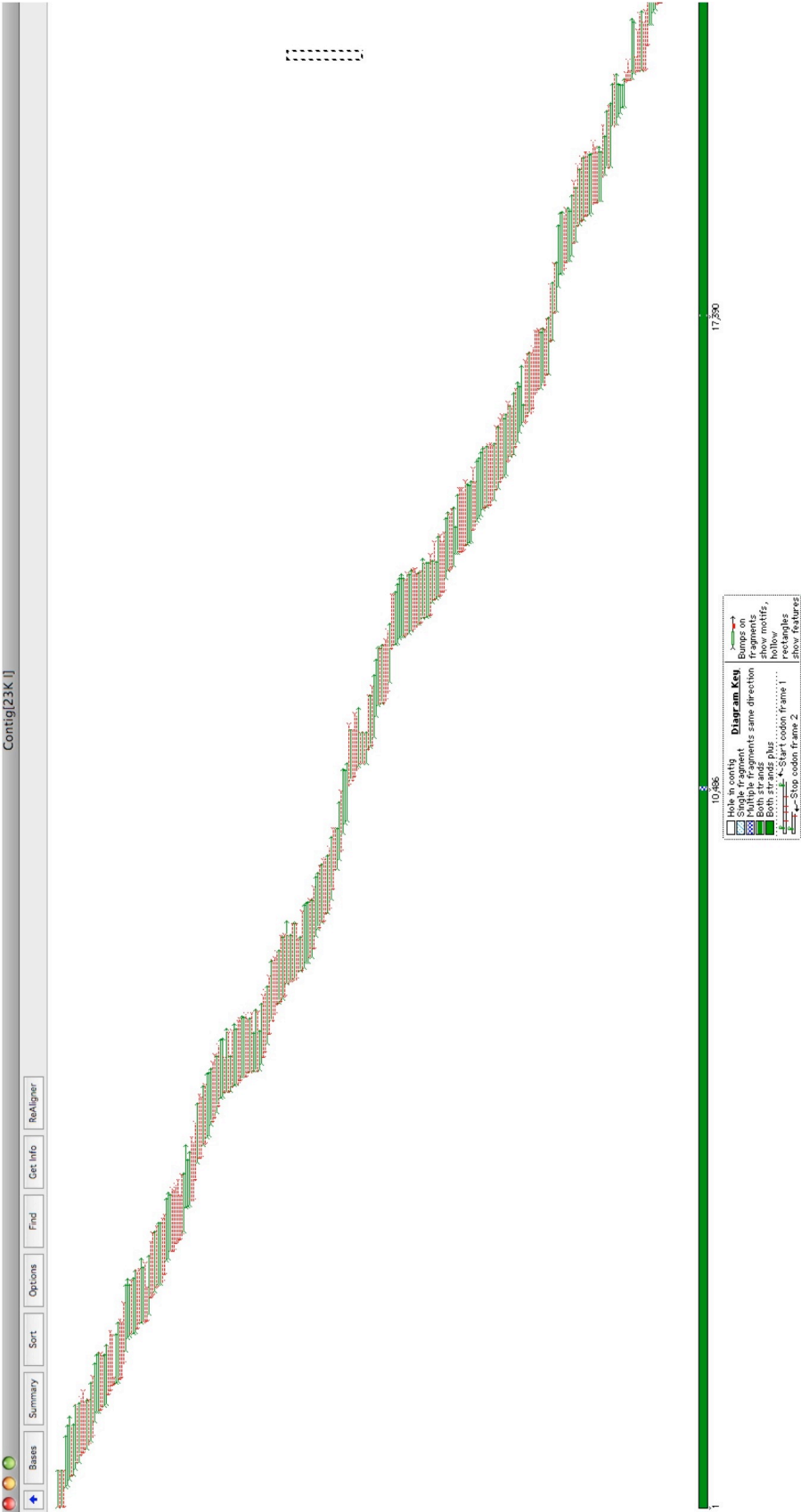
Figure III-8. Contig assembly of shotgun sequence using Sequencher.

- A. Contig of BAC 178-C20 containing the Omny-Chit.01 sequences. The full length of the Contig is 86,590 bp containing 1282 sequences.
- B. Contig 23K I for BAC 228-I23 containing the Omny-Chit.02 sequences. The full length of the Contig is 23,139 bp containing 325 sequences.

A 178-C20 Contig containing Omny-CHIT.01



B 228-I23 Contig containing Omny-CHIT.02

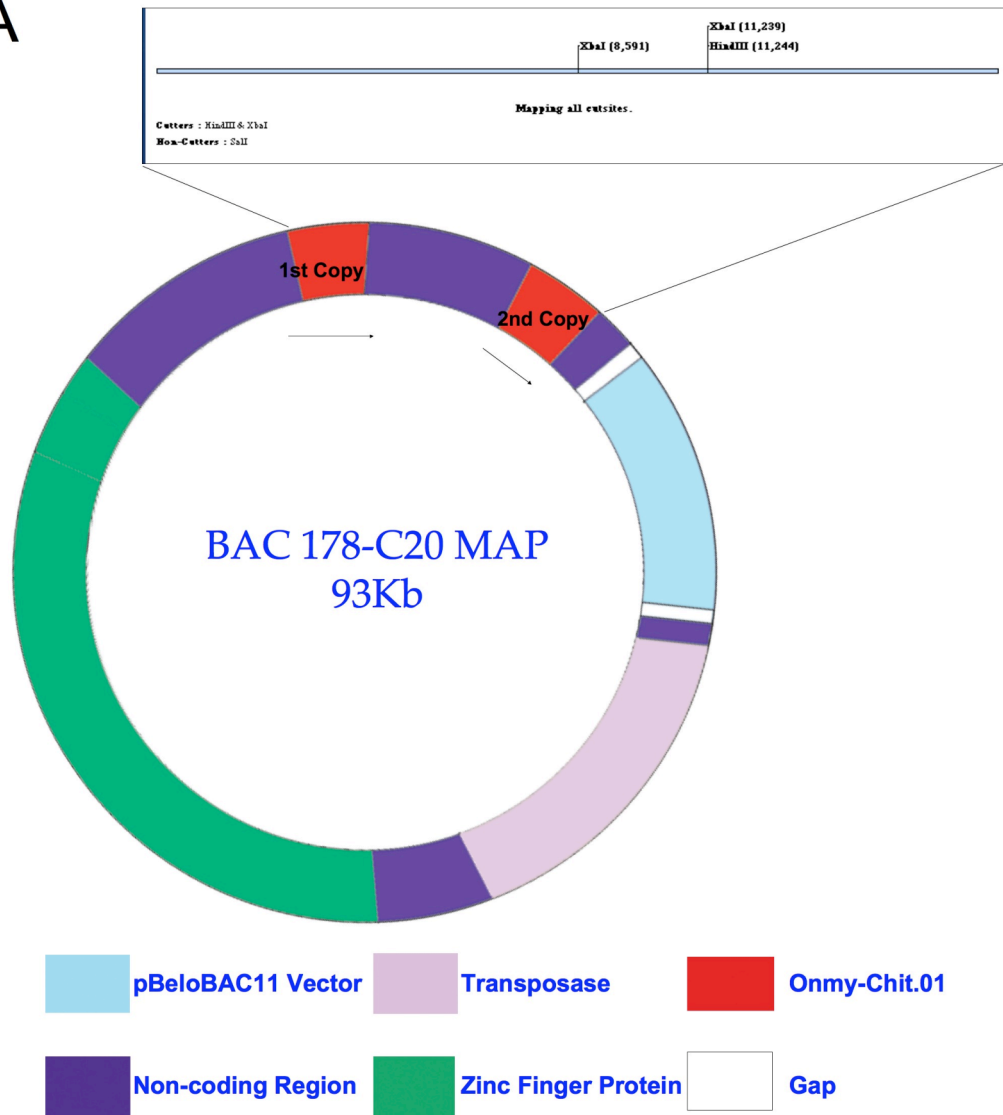


Figure

III-9. BAC clone maps.

- A. BAC 178-C20's total length is around 93Kb with two major Contigs assembled. The largest Contig is around 86Kb containing two copies of Onmy-Chit.01 9Kb apart. The three restriction enzyme cutting sites in this 9Kb region are illustrated on inset. HindIII has one cutting sites, Xba I has two, SalI does not have cutting sites. This result is consistent with BAC clone southern blotting results (Figure III-5). The other contig is 7kb pBeloBAC11 vector. These two Contigs have not been connected.
- B. BAC clone 228-I23. Five major Contigs were recovered from the assembly. The Onmy-Chit.02 gene was contained in Contig 23K I.
- The arrows indicate the direction of translation for the predicted open reading frame.

A



B

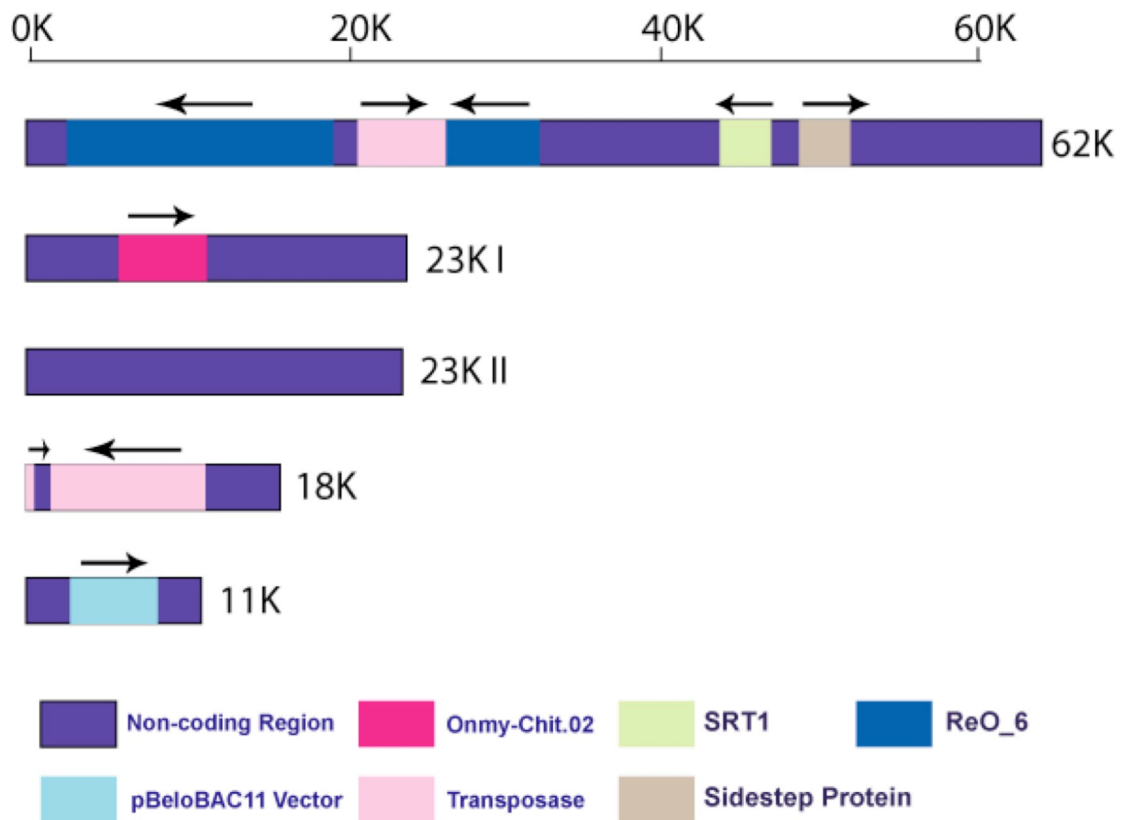


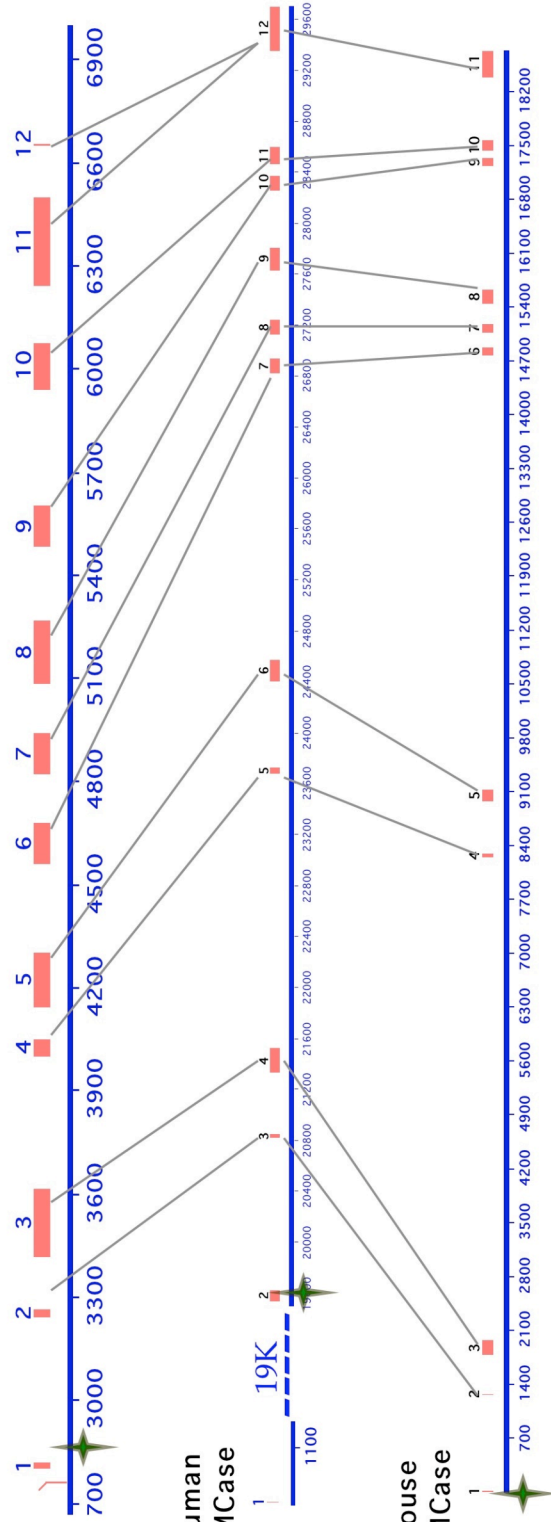
Figure III-10. Comparison of the rainbow trout Chitinase gene structures to human and mouse.

A. Onmy-Chit.01 gene structure compared to the human and mouse AMCase genes.

B. Onmy-Chit.02 gene structure compared to the human and mouse Chitotriosidase genes. The grey lines connect homologous exons (Table III-6). The green star indicates the translation start site for each gene.

A

Onmy-Chit.01



B

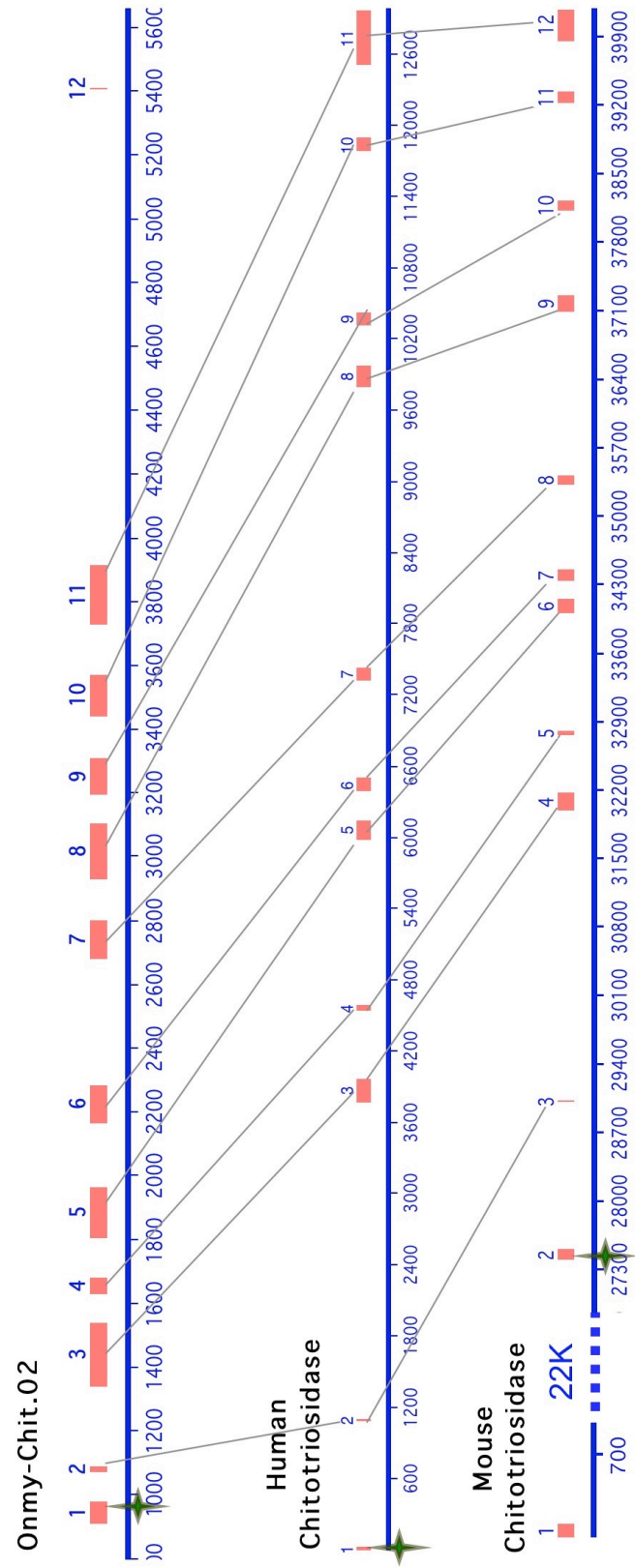


Figure III-11. Comparative sequence analysis for vertebrate Chitinase genes. MVista37 graphical representation of a Multi-Lagan36 multiple sequence alignment of vertebrate Chitinase loci. Conserved regions are displayed relative to their positions in the Onmy-Chit.01 first copy (horizontal axis). Segments that show more than 50% sequence identity (indicated on the vertical axis) at the nucleotide level over a 100-bp window, are highlighted in pink (noncoding regions). The pink square with blue number indicates exons of Onmy-Chit.01. All aligned sequences contain the coding region and 2000 bp upstream of translation start site.

Human CHIA,*Homo sapiens* AMCase ,NT_019273.18;
Human Chit,*Homo sapiens* Chitotriosidase ,NP_003456.1;
Mouse CHIA,*Mus musculus* AMCase ,NW_001030731.1;
Mouse Chit,*Mus musculus* Chitotriosidase ,NW_001030662.1;
Rat CHIA,*Rattus norvegicus* AMCase ,NW_047627.2;
Rat Chit,*Rattus norvegicus* AMCase ,NW_047395.1;
Fugu CHI,*Takifugu rubripes* Chitinase,from scaffold_60;
Tetradon CHI,*Tetraodon nigrovirdis* Chitinase ,CR655545;
Zebrafish CHI1,*Danio rerio* Chitinase 1,BC045331;
Zebrafish CHI2,*Danio rerio* Chitinase 2,BC044549;
Zebrafish CHI3,*Danio rerio* Chitinase 3,BC045887.

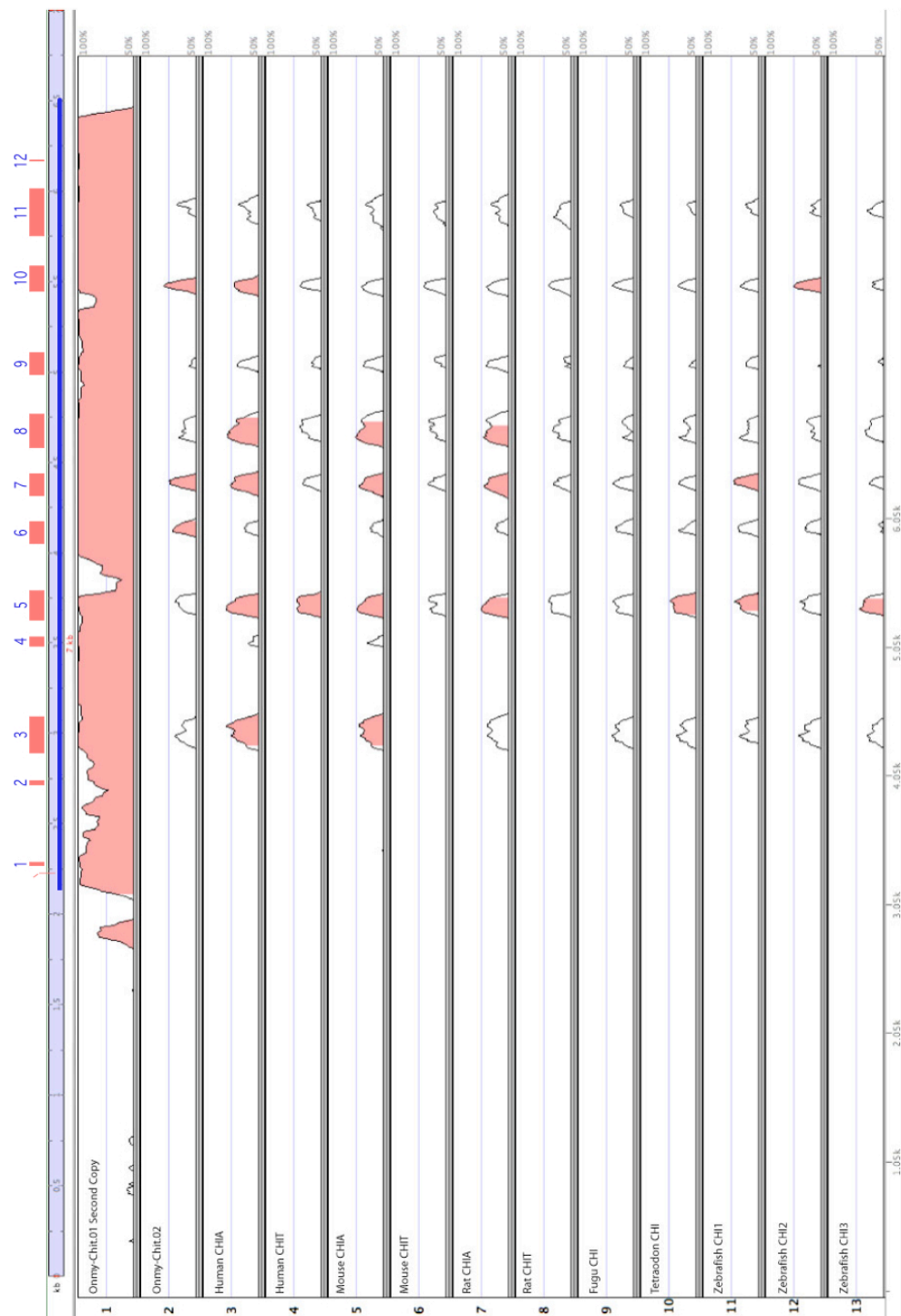


Figure III-12. Comparison of evolutionary conserved regions (ECR) in vertebrates genomes using the human Chitinases genes as queries. The plots, derived from the DCODE Comparative Genomics Center ECR genome browser (<http://ecrbrowser.dcode.org>). Color codes: blue, coding exon; yellow, UTR; pink, intron; Red, intergenic element; green, repetitive element.

A. AMCase

B. Chitotriosidase.

[illegible]

B

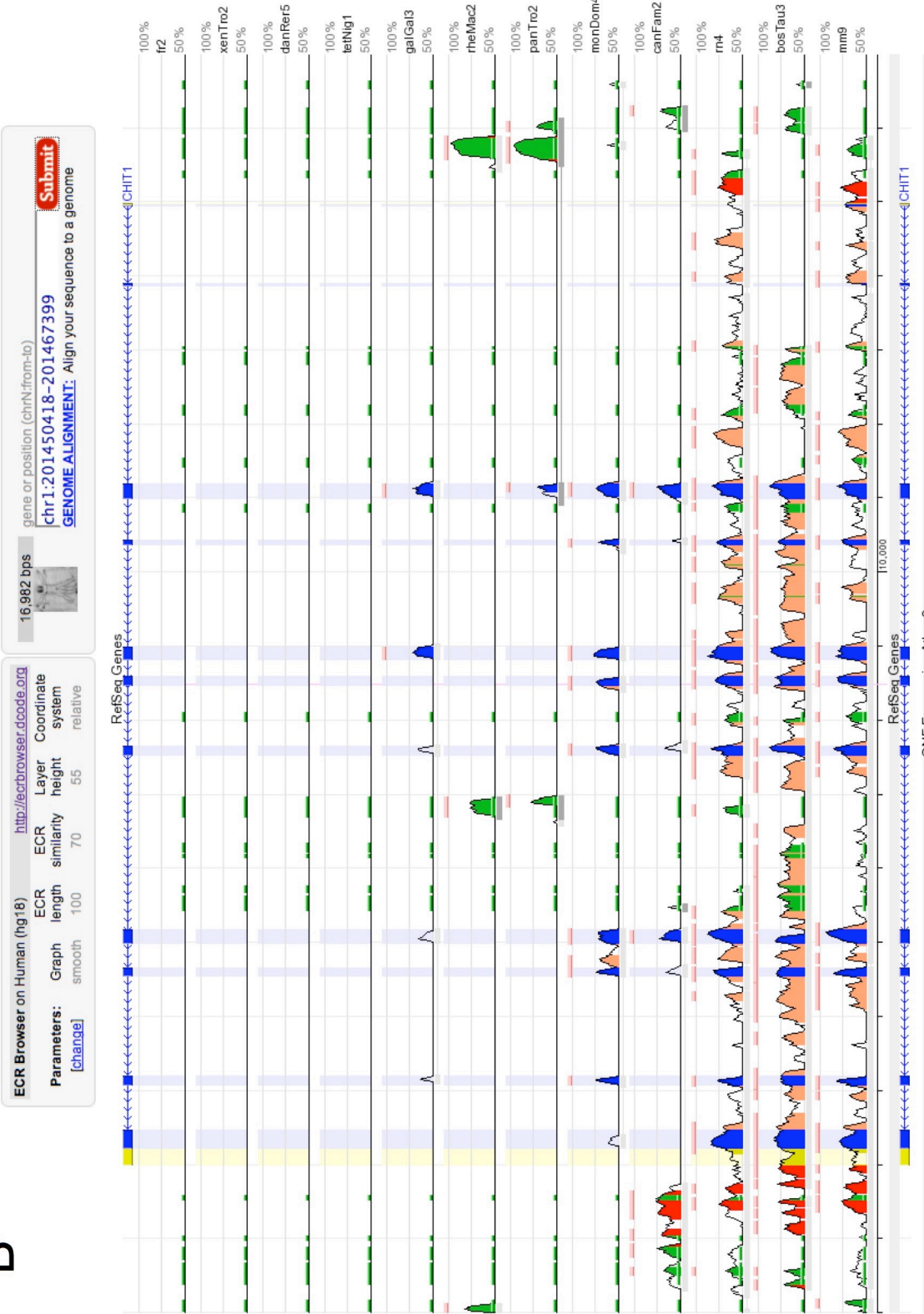


Figure III-13. ECR browser results for zebrafish Chitinase genes. In order to investigate ECRs of Chitinase genes in fish, I aligned Onmy-Chit.02 gene with zebrafish genome. The alignment results were used as the base to search ECRs among all the genomes available in the database.

A. Only the Fugu genome showed three ECRs in intergenic regions. The three ECRs are named A, B and C. Color codes: blue, coding exon; yellow, UTR; pink, intron; Red, intergenic element; green, repetitive element.

B. Closer examination of ECR C of Figure III-13. The plots, derived from the DCODE Comparative Genomics Center ECR genome browser (<http://ecrbrowser.dcode.org>). Color codes: blue, coding exon; yellow, UTR; pink, intron; Red, intergenic element. The red peak shows the ECR C.



Figure III-14. Dynamically overlay transcription factor binding site prediction with the conservation profile and perform clustering of ECRs of Figure III-13. A) ECR A; B) ECR B; C) ECR C.

ECR A

Zebrafish genome location: chr11:21206146-21206367

Length: 222 bps

Identity: 73.9%

Sequences

> danRer5 chr11:21206146-21206367 222bps GC:43.7%

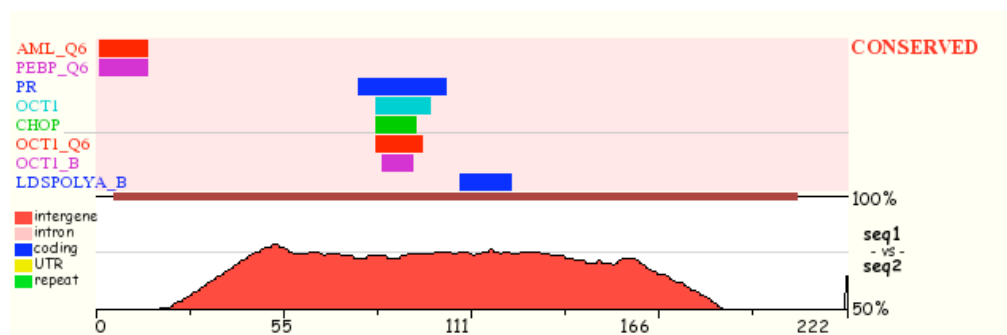
TAGCTTAGTGGTTAACACTTTTGCCTCACAGTAAGAAGGTCGCTGGTTCGAGTCCTGTCT
GGGCCAGTTGGCATTCTTTGTGTGGAGTTTGCATGTTCTCGCTTTTTTCATGTGGGTTTCC
TCCAGGTGCTCCGGTTCTTTCCACAAGACATGTGGTACAGGTGAATTGAATAAACTAAAT
TGGCCGTAGTGTATGTGTGTAATGAGTTTGTATGGATGTTT

> fr2 chrUn:322929473-322929709 237bps GC:52.7%

TAGTGTGGTGGTTAGCACTGTTGCCTCACAGCAAGAAGGCCCGGGTTCGATCCCCGGTT
GGGACTGAGGCTGGGGACTTTCTGTGTGGAGTTTGCATGTTCTCCCTGTGCCTGCGTGGG
TTCTCTCCGGGTACTCCGGCTTCTCCACAGTCCAAAGACATGCATGATTGGGGATTAG
GCTAATTGGAACTCTAAATTGCCCATAGGTTTGAGTGTGAGAGAGAATGGTTGTTT

Table III-11.Nine identified conserved transcription factor binding sites (TFBS) in ECR A.

No.	TFBS name	Strand	Binding Region	Sequence
1	AML_Q6	+	2-16	agcttAGTGGTtaac
2	PEBP_Q6	-	2-16	agcttAGTGGTtaac
3	PR_01	+	78-104	tgtgtggAGTTTGCATGTTCTcgcttt
4	OCT1_Q6	+	83-96	ggaGTTTGCATGtt
5	CHOP_Q6	-	83-95	ggagTTTGCAtgt
6	OCT1_Q6	-	83-97	ggaGTTTGCATgttc
7	OCT1_Q2	-	85-99	agttTGCATGTTc
8	OCT1_B	-	85-94	aGTTTGCATg
9	LDSPOLYA_B	+	108-123	catgTGGGTttcctcc



Dynamically overlay TFBS prediction with the conservation profile and perform clustering of ECR A.

ECR B

Zebrafish genome location: chr11: 21207170-21207497

Length: 328 bps

Identity: 75.0%

Sequences

> danRer5 chr11:21207170-21207497 328 bps GC:49.1%

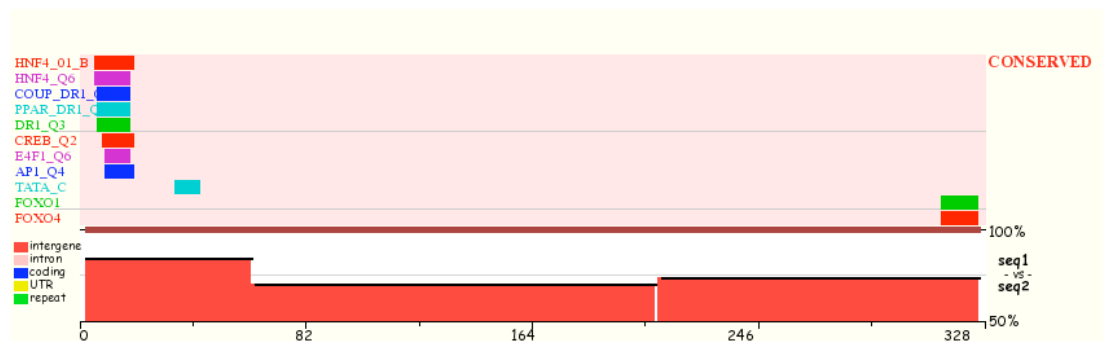
CAGGGCTGCCCTTTGTCACCAATTCTGATCATAATTTTATAGGCAGAATTTCAAAGCGC
AGCAGGGGTCCGGTTCGGGGACCACAGGATCTCATCTCTGTTATTAGCAAATGATGTTGT
TCTGTTGGCTTCATCGAACATGGACCTTCAGCATGCACTGGGGTGGTTTGCTGCCGAGTT
TAATGCGGCTGCGATGAGAATCAGCACCTCCCAAGTCCGAGGCCATGGTGATCCACTAGA
AAATGGTGATTTGCCATCTCCAGGTTGGAGGACAGTCCTTACCCCAGGAAGAGGAGTTCT
AGTATTTGGGGTTTTGTTCACAAGTGA

> fr2 chrUn:337363144-337363481 338 bps GC:52.1%

CAGGGCTGCCCTTTGTCACCGATTCTGTTCATAACTTTTATGGACAGAATTTCTAGGTGC
AGTCATGGTGTGAGGGGGTCCGGTTTGGTGACCTCAGGATCGGGTCTTTACTCTTTGCA
GATGATGTGGTCCTGTTGGCGTCATCGGCCCGTGACCTCCAACGATCAGTGGATCAGTTC
GCCGCCGCATCTGAAGCGGCTGGGATAAAAAACAGCACCTCCAAATCCGAGGCCATGGTT
CTCAACCAGAAAAAGGTGGAGTGCCTTCTCCGGGTCAAGGAGAAGATCCTGCCCCAAGTG
GAGGAGTTCAAATACCTTGGGGTTTTGTTCACAAGTGA

Table III-12. Eleven identified conserved transcription factor binding sites (TFBS) in ECR B.

No.	TFBS name	Strand	Binding Region	Sequence
1	HNF4_01_B	-	6-20	cTGCCCTTTGTCAcc
2	HNF4_Q6_01	-	6-19	cTGCCCTTTGTCAc
3	COUP_DR1_Q6	+	7-19	TGCCCTTTGTCAc
4	PPAR_DR1_Q2	+	7-19	tGCCCTTTGTCAc
5	DR1_Q3	-	7-19	TGCCCTTTGTCAc
6	CREB_Q2	-	9-20	cccTTTGTCAcc
7	E4F1_Q6	+	10-19	ccTTTGTCAc
8	AP1_Q4	-	10-20	ccttTGTCaacc
9	TATA_C	-	35-44	tTTTTATAGg
10	FOXO1_02	+	312-325	gttTTGTTCACaag
11	FOXO4_02	+	312-325	gttTTGTTCACaag



ECR C

Zebrafish genome location: chr11: 21233394-212336

Length: 207 bps

Identity: 73.4%

Sequences

> danRer5 chr11:21233394-21233600 207bps GC:42.0%

GTAGTTAGCAATGTTGCCTCACAGAAAGAAGGTCGCAGGTTTAAATCCCTGATGGAATGG
GTCAGTTGGCATTCTGTGTGGAGTTTGCATGTTCTCCCATGTTACGTGGGTTTGCCC
CACTGTCCAAAGACATGCGGTATAGATGAATAAAATTAATACTAAATTGGTCTTAGTGTATG
TGTGTAATGAGTGTGTATGGATGTTT

> fr2 chrUn:322929480-322929713 234bps GC:53.0%

GTGGTTAGCACTGTTGCCTCACAGCAAGAAGGCCCGGGTTCGATCCCCGGTTGGGACTG
AGGCTGGGGACTTTCTGTGTGGAGTTTGCATGTTCTCCCTGTGCCTGCGTGGGTTCTCTC
CGGGTACTCCGGCTTCCTCCACAGTCCAAAGACATGCATGATTGGGGATTAGGCTAATT
GGAAACTCTAAATTGCCCATAGGTTTGAGTGTGAGAGAGAATGGTTGTTTGTCT

Table III-13.Six identified conserved transcription factor binding sites (TFBS) in ECR C.

No.	TFBS name	Strand	Binding Region	Sequence
1	RFX_Q6	+	11-19	atGTTGCct
2	OCT1_Q5	+	81-94	ggaGTTTGCATGtt
3	CHOP_Q1	-	81-93	ggagTTTGCATgt
4	OCT1_Q6	-	81-95	ggaGTTTGCATgttc
5	OCT1_Q2	-	83-97	agttTGCATGTTc
6	OCT1_B	-	83-92	aGTTTGCATg

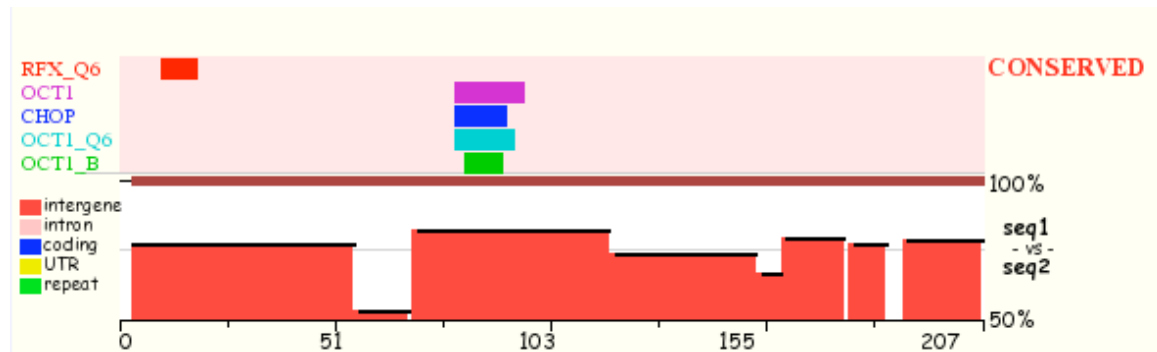


Figure III-15. Schematic overview of the synteny of mouse locus 3F3 with human 1p13. On the left-hand side, mouse chromosome 1 with a part of locus 3F3 is indicated. On the right-hand side, the human syntenic region 1p13 is shown. The orientation and position of a few genes in the direct neighborhood of the mouse and human Chitotriosidase gene are depicted. The CHIA: acid mammalian Chitinase is depicted in pink shadow; CHI3L2: Chitinase 3-like protein 2, also named Bclp2 in mouse is indicated by red underline.

	Identity Scores (%)			
Predicted Protein	Mouse AMCase	Mouse CHI3L2	Human CHI3L2	Human AMCase
Mouse AMCase	100	59.7	38.3	80.7
Mouse CHI3L2	72.4	100	38.8	60.3
Human CHI3L2	54.4	57.8	100	39.5
Human AMCase	89.3	71.2	55.1	100
	Similarity Scores (%)			

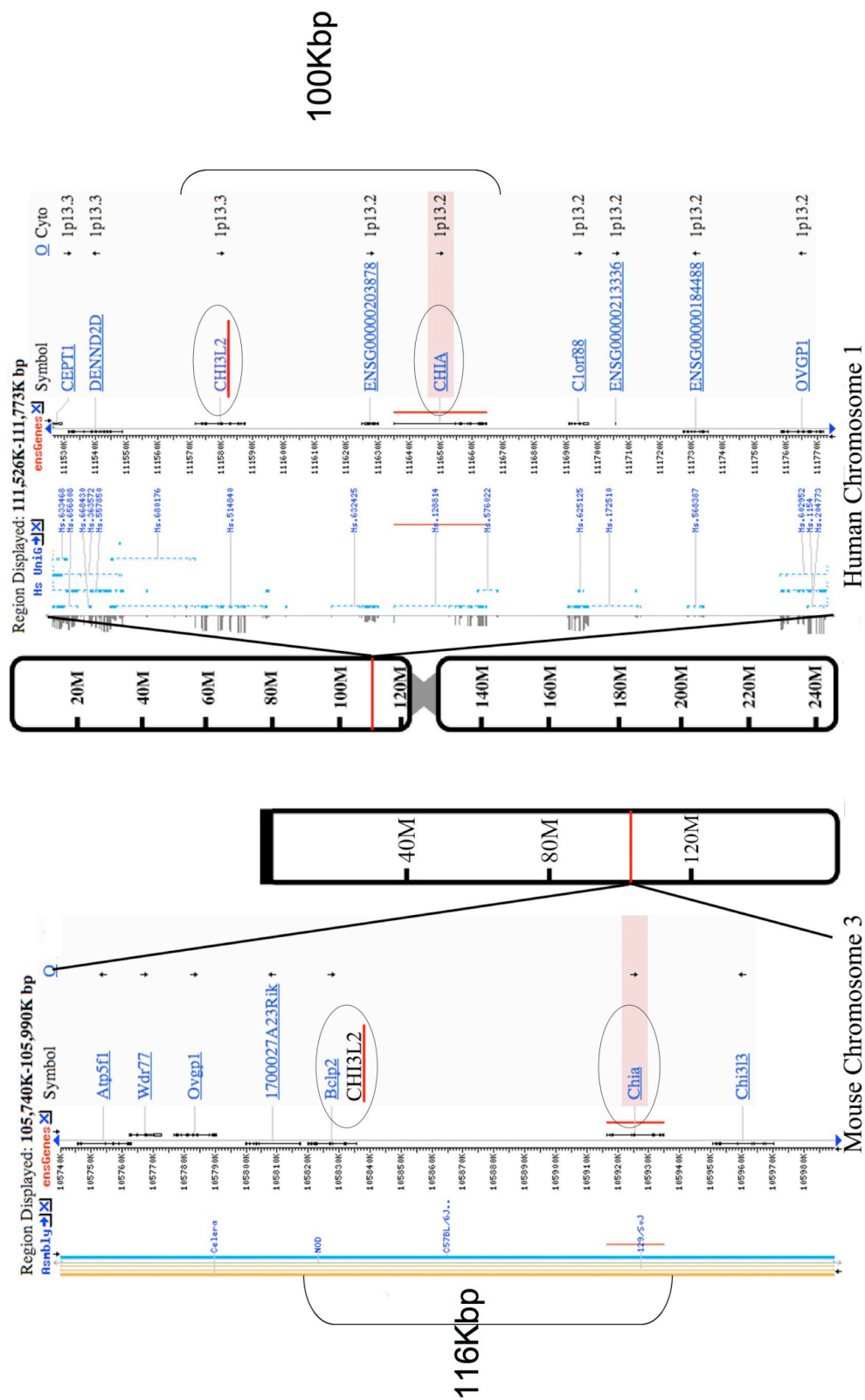


Figure III-16. Schematic overview of the synteny of mouse locus 1E4 with human 1q32. On the left-hand side, mouse chromosome 1 with a part of locus 1E4 is indicated. On the right-hand side, the human syntenic region 1q32 is shown. The orientation and position of a few genes in the direct neighborhood of the mouse and human Chitotriosidase gene are depicted. The genes of members of the Chitinase protein family are depicted in dark gray arrows, whereas the other genes are indicated with light gray arrows. The genes are BTG2, Btg2: B-cell translocation gene 2; CHIT1: Chitotriosidase; CHI3L1, Chi3l1: cartilage glycoprotein 39; MYBPH, Mybph: myosin-binding protein H; ADORA1, Adora1: adenosine A1 receptor; LOC388729Table III-: gene coding for the human hypothetical protein with GenBank Accession Number: [XP373882](#). Cited from [92].

	Identity Scores (%)			
	Human Chitotriosidase	Mouse Chitotriosidase	Human CHI3L1	Mouse CHI3L1
Human Chitotriosidase	100	74.7	43.2	41.2
Mouse Chitotriosidase	83.7	100	41.7	39.2
Human CHI3L1	59.6	57.9	100	72.9
Mouse CHI3L1	58.6	55	84.9	100
	Similarity Scores (%)			

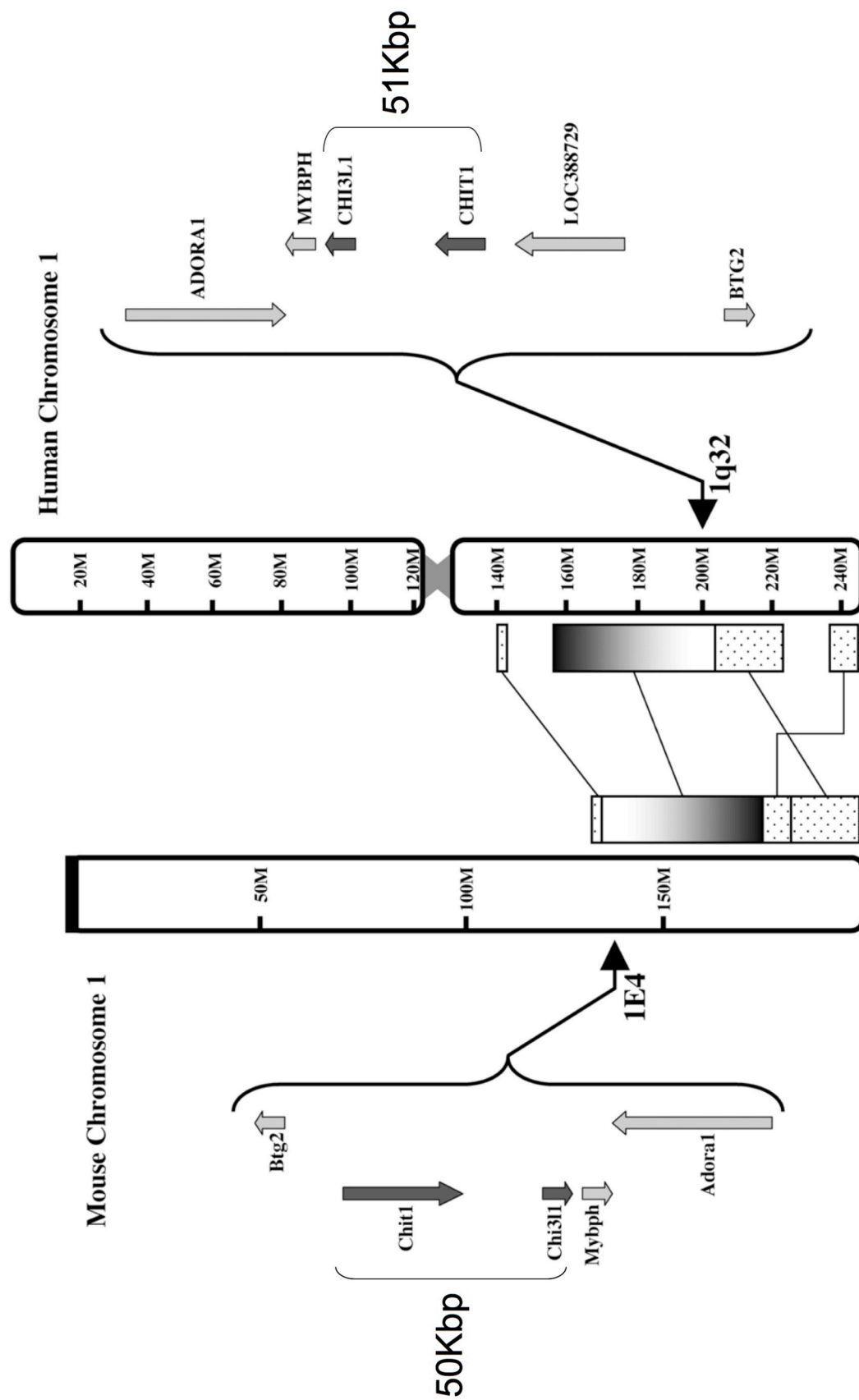
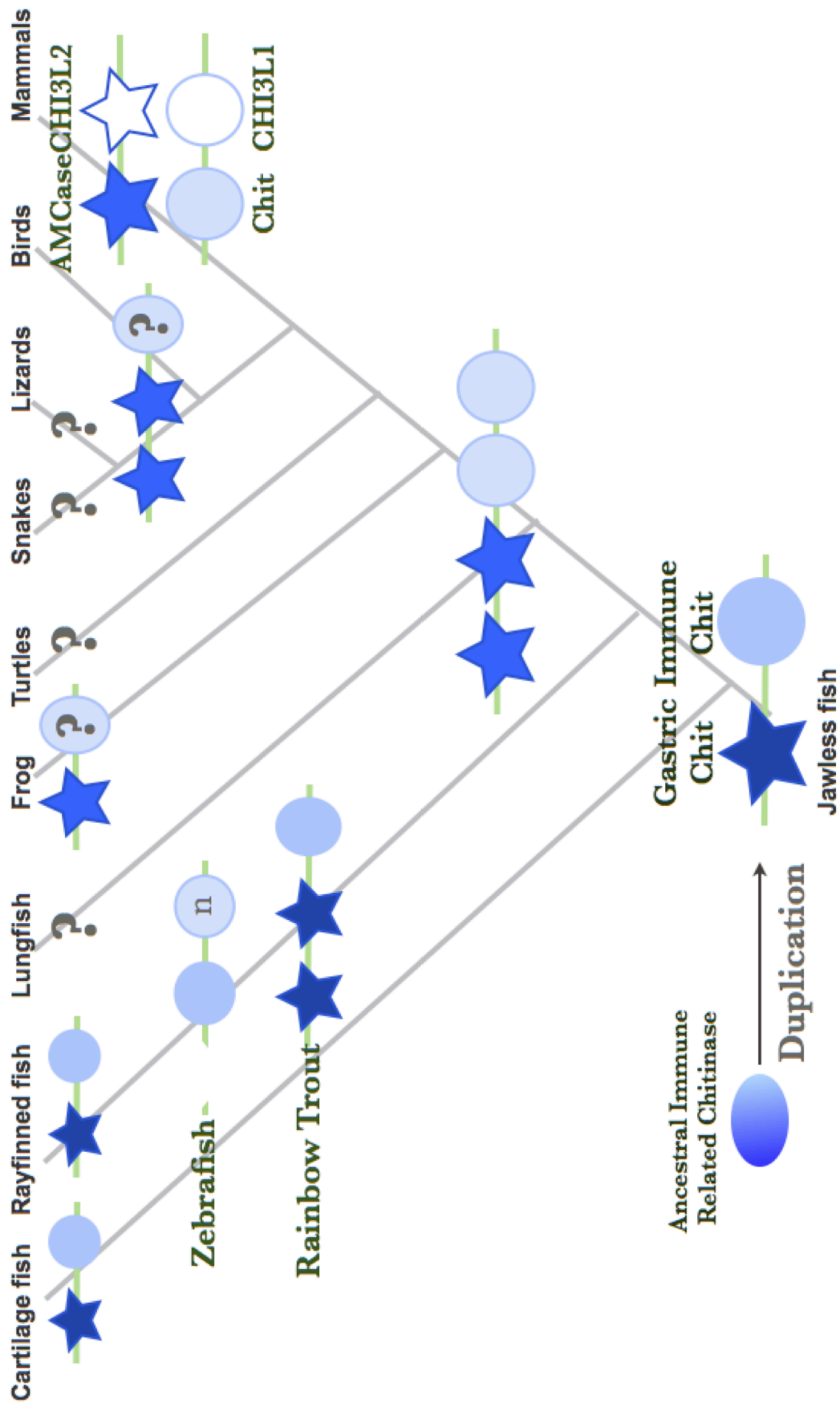


Figure III-17. Pictorial model illustrating the evolutionary fate of duplicated genes encoding chitinase. Color gradient denotes the mutation process of the gene. The darker the color is, the more ancestral the gene is. White symbols indicate the mutations giving rise to loss of catalytic activity. Question marks indicate lack of data to make a prediction.



Chapter IV *In vivo* and *In vitro* stimulation Of Rainbow Trout Chitinases

IV.A. Introduction

In the previous two chapters, I discussed the cellular expression and possible regulatory elements for the two chitinase genes Onmy-Chit.01 & 2 found in the rainbow trout (*Onchorhynchus mykiss*) genome. Onmy-Chit.01 was expressed extensively in the gastric portion of the stomach. Onmy-Chit.02 was expressed at much lower levels in the immune cells of the myeloid cell lineage. However, it is not clear which stimulants will induce or whether induction is possible for cellular expression *in vivo* and *in vitro*. In mammals, chitotriosidase mRNA accumulation is induced by IFN- γ , TNF- α , and LPS[148]. In humans chitotriosidase is used as a marker for stimulated macrophages under certain condition. On some occasions it is the main protein secreted, representing approximately 1% of the total protein secretion [42].

The substrate of chitinase - chitin and its deacetylated derivative- chitosan, are frequently used as immunostimulants[5, 16], as mentioned in the introduction chapter. Both have been used to prime the macrophages in mice. Chitin and chitosan can boost host immune systems in fungi-infected mice[16]. Phagocytosable chitin (1-10 μ m) activates the alveolar macrophages and spleen cells, which produced in response IL-12, TNF- α and IL-18 [17, 18].

In addition to being the load bearing fiber in the exoskeletons of many invertebrates, chitin is an important part of the fungal cell wall, even though not all fungi have chitin as their structural element. To mimic fungal infection, zymosan -an insoluble yeast cell wall

particle, is widely used as immunostimulant in both *in vivo* and *in vitro* studies to activate the immune system and stimulate protective host response. Zymosan particles consist of a variety of components (including glucans, mannans, chitin, protein, and lipids), and it is believed that the β -glucan is the biologically active constituent. However, while Shibata et al. [18] observed that zymosan can induce production of IFN- γ , IL-12 and TNF- α of alveolar macrophages, the purified β -glucan was inactive. They claimed that particular terminal sugar residues in the microbes, such as mannose and chitin, are recognized and phagocytosed by macrophages preferentially to induce the above cytokines.

Human chitotriosidase was found to react with fungi, inhibiting growth of *Candida neoformans*, causing hyphal tip lysis in *Mucor rouxii* and preventing the occurrence of hyphal switch in *Candida albicans*[105]. In the guinea pig Overdijk et al. showed that intravenous infection of guinea pigs with the fungus *Aspergillus fumigatus* resulted in an increased level of chitinase in serum and tissues[175]. Within the examined tissues, spleen showed extremely high expression compared with heart and brain[175].

In order to explore the possible stimulants of rainbow trout chitinase, whole animal responses to wounding and fungal infection were examined. In addition primary cultured adherent cells (presumptive macrophages) from trout head kidney were stimulated with bacterial lipopolysaccharide (LPS), zymosan, chitosan, and chitin particles. Changes in chitinase mRNA expression levels were quantified using qPCR.

IV.B. Material and Methods

IV.B.1. *In vivo* stimulation

Pure strains of the aquatic fungal pathogen (*Saprolegnia parasitica*) were obtained from the American Type Culture Collection (ATCC, # 22284), Rockville, Maryland. The fungi were cultured on corn meal agar (CMA) slants, covered with heavy mineral oil, and stored at $4 \pm 2^{\circ}\text{C}$ until used. Before inoculation, fungi were sub-cultured in L-15 media and grown for 4 days. For inoculation, 1 ml of fungi-containing L-15 medium was inoculated to autoclaved sunflower seeds (halved) in a 50 ml flask. The infected sunflower seeds were put into three large histology cassettes and placed in 60 gallon tank a containing rainbow trout treated as described below.

Trout was anesthetized with MS 222 and abraded with Dremel power tool; scales were removed between adipose fin to back of dorsal fin – about 1 cm wide. Abraded trout were exposed in fungal inoculated tank for 4 hrs. After exposure, they were randomly put into a 20 gallon tank containing dividers (3 fish/ tank).

Table IV-1.Trial 1-1 set-up. Three fish from each group was sampled at Day 2 and the remaining fish at Day 8

Group	Total Fish	Treatment
Control	7	No treatment
Abraded	10	Abraded
Abraded+infected	10	Abraded and infected

IV.B.2. Production of Tritiated Chitin

Acetyl-[^3H]-chitin was prepared by reacylation of chitosan (practical grade, from crab shells, Sigma Chemical) with tritiated acetic anhydride (NEN) as described by Molano, et al. [125] and modified by Cabib and Subrlati [176] The chitin was suspended

in water at a final concentration of ~15 mg/ml and stored at 4°C. The specific activity of the reacylated chitin was determined by the consecutive degradation of the substrate into GlcNAc monomer by chitinase (*Serratia marcescens*, 14.3 mg/ml [0.215 units/ml] in 0.1 M Na₂PO₄, pH 6.0) and β -glucuronidase (*Helix pomatia*, 12.9 mg/ml [6592 units/ml] in the same buffer as *S. marcescens*), both from Sigma Chemical Company. Total GlcNAc was determined based on a standard curve of NAG (1, 0.44, 0.88, 2.2, 3.3, 4.4, 5.5, 6.6 and 8.8 μ g of NAG; 0, 1, 2, 5, 7.5, 10, 12.5, 15, 20 μ L of a 2 mM stock solution of NAG; R²=1.0) using a microtiter colorimetric assay. Controls were run concurrently (zero chitin and zero enzymes) and were deducted from the final yield.

IV.B.3. Chitinase and lysozyme activity assays

Chitinase activity was assayed using the tritiated chitin produced (Chapter 2). The 100 μ L assay solution consisted of 5 μ L of 1 M ammonium acetate pH 4.5, 15 μ L chitin suspension, assay sample and water. The assays were incubated in 1.5 mL microfuge tubes in an Eppendorf thermomixer. The constant action of the thermomixer kept the chitin constantly suspended and available for enzymatic degradation. The enzymatic reaction was stopped and insoluble chitin was precipitated with 300 μ L of 10% trichloroacetic acid (TCA). This mixture was centrifuged for 5 minutes at 14,000 g and 200 μ L of the supernatant was carefully removed so as not to disturb the chitin pellet and the tritium of that solution was measured. The basic unit of this assay is μ g GlcNAc released/hour.

Lysozyme activity was determined by the method of Grinde et al.[177] modified to a microtiter format. *Micrococcus lysodeikticus* was dissolved in 40 mM sodium phosphate buffer, pH 6.5. Test solution was added (3-20 μ L) to substrate solution for a final volume

of 200 μ l. Softmax (Molecular Devices) software was used to collect and analyze the data. The software was set up to make a kinetic reading (absorbance at 650 nm was read and recorded every 10 seconds for 5 minutes) at 25 °C and the plate was shaken between each reading (to keep the *M. lysodeikticus* suspended) but not before the first reading so that the first reading is at the beginning of the enzymatic reaction. The instrument reduction settings were –0.25 (minimum O.D.) and 0.25 (maximum O.D.). Units of lysozyme per volume were determined by measuring the maximum velocity (the steepest slope of the decreasing absorbance) over the five minute assay period. Assays were run in triplicate and the raw data (U) was the absolute value of the negative maximum velocity. Specific lysozyme activity is calculated by determining the U per milliliter and then dividing by total protein (mg/ml). It is expressed as U per milligram of total protein

IV.B.4. Protein Assay

Total protein was determined with a BCA kit (Pierce) according to the manufacturer's instructions using bovine serum albumin (0-20 μ g) as the standard. Samples (volumes of 0-100 μ L) were loaded to a microtiter plate and the volume was brought up to 100 μ L with water. The colorimetric reagent (5 parts Reagent A, 4.8 parts Reagent B, 0.2 parts Reagent C) was added to each test solution for a final volume of 200 μ L. The plate was shaken for 30 seconds to ensure proper mixing, incubated at 60°C for 1 hour and the absorbances read at 562 nm. The raw spectrophotometric data was analyzed using Softmax (Molecular Devices) software. Standard curves were plotted using a quadratic fit.

IV.B.5. *In vitro* primary culture of head kidney adherent cells and subsequent stimulation

Rainbow trout were sacrificed by over-anesthetization in MS222 and the head kidneys were dissected and placed in sterile 200 μ m nylon mesh bags (Becton Dickinson). The tissue was squeezed through the bag using sterile forceps into Leibovitz L-15 medium (Invitrogen) containing 10% heat inactivated fetal calf serum (Gibco); heparin (10 units/ml), penicillin (100 U/ml)/and streptomycin (100 μ g/ml). Cells were counted with a hemacytometer, diluted to a final concentration of 1.0×10^7 cells/ml and plated on 35 mm culture plates coated with poly-lysine (Becton Dickinson) at a density of 2.5×10^8 cells/plate. The cells were held in an incubator at 15 °C. Nonadhering cells were removed after 24 h with washing in sterile media and new media (as above) was added and the cells were incubated for an additional 4 days.

After a total of 5 days culture, plates were stimulated with various presumptive regulating agents. Following stimulation, the medium was decanted and replaced with 2 ml of Tri Reagent (Molecular Research Center, Inc.) per well after certain time. Total RNA was extracted using Tri Reagent according to the manufacturer's protocol. The RNA was reverse transcribed using AMV reverse transcriptase (Promega) and random primers. The cDNA was used as a template for real time PCR with probe designed to selected gene sequences. Fluorescence was measured at the end of every extension step. C_T (threshold cycle) values were calculated and normalized for each gene against those obtained for its RNA.

Stimulants Used:

Control: PBS

β -D-Glucan (Sigma #G6513, β -D-Glucan from barley),

Zymosan (Sigma #Z4250, Zymosan A from *Saccharomyces cerevisiae*)

Chitin (Sigma #C-8908, poly-N-acetylglucosamine purified powder from shrimp shells)

Chitosan (Chitosan 1000, Wako Pure Chemical Industries, Ltd.)

LPS (Sigma # L2630, Lipopolysaccharides from *Escherichia coli* 0111:84)?

Table IV-2. Inducers Tested and Concentration used.

Stimulant	Sample	Concentration
1	PBS	1X
2	Chitin	300 μ g/ml
3	Chitosan	300 μ g/ml
4	LPS	10 μ g/ml

Sample collection time-course

2, 4, 6, 8, 10, 16 and 24 hour after stimulation

--Trial 1-2:

Chitin and Chitosan were suspended in PBS at 10 mg/ml, and sonicated at 25% output power twice for 5 mins each with a Branson Sonicator (sonifier 250, Branson Ultrasonics, Danbury, Conn.). The particle suspension was filtered through a 400/2800 mesh to obtain small particles (1~10 μ m)[4]. Each suspension was exposed under UV light for 2 hrs to sterilize.

Table IV-3. Inducers Tested and Concentration used.

Stimulant	Sample	Concentration
1	PBS	1X
2	Chitin<10 μ m	300 μ g/ml
3	Chitosan<10 μ m	300 μ g/ml
4	β -Glucan	25 μ g/ml
5	Zymosan	25 μ g/ml
6	LPS	10 μ g/ml

Sample collection time-course - 8, 16, and 24 hours after exposure.

IV.B.6. qPCR Taqman assay and data analysis

For TNF (Genbank accession AJ277604), a 405bp amplicon from TNF ORF was amplified from rainbow trout head kidney library. For Onmy-Chit.02, a amplicon containing the full sequence of Chit02 open reading frame (ORF) was obtained. The amplicon was ligated into pCR3 vector (Qiagen). The ligations were transfected and plasmids were extracted as described in Chapter II. The concentration was measured by spectrophotometry using a Nanodrop, and a series dilution was made. On each running plate, the series dilution of plasmid standard was run with samples.

The position of probes used for qPCR assay were illustrated in Figure IV-4.

	Name	Sequence	Start	End
1	TNFqPCRF	GCATGGAAGACCGTCAACG	314	332
2	TNFqPCRR	TGGGTATCTTTTGCACCAATGA	388	367
3	TNF qPCR Probe	TGCAGGATGAAATTGAGCCTCAACTGGA	334	361
4	TNFqPCR Plasmid Template Forward	AACAGCAGTATGGAGGGGTATGC	132	154
5	TNFqPCR Plasmid Template Reverse	AGTGTGTGGGATGAGGATTTGG	577	536

IV.B.7. Histology of the wound site

Histology section and staining was performed as described[178].

IV.B.8. Statistical Data Analysis

Two way (Day and Treatment) ANOVAs were performed using Aable (GIGAWIZ, Version 2.4.2 R) after checking for homogeneity of variance and normality.

IV.C. Results

IV.C.1. In *vivo* response to wounding and fungal infection

Preliminary studies indicated that maximum response to wounding occurred around eight days post-treatment. Accordingly, I chose Day 2 and Day 8 for sampling times. As shown in Figure IV-1, abrasion of rainbow trout skin caused massive infiltration (Figure IV-1-B) of the wound site by macrophages with extensive fungal growth. Analysis (ANOVA) of plasma lysozyme and chitinase activity found no changes with Treatment (Control, Abraded and Abraded and Infected) or Day (Day 2 and Day 8 post treatment) for plasma lysozyme activity. However, plasma chitinase activity at Day 2 was significantly elevated for the abraded treatment (Figure IV-2A) (Tukey-Kramer, $P = 0.002$ and $P = 0.003$, respectively) relative to control and abraded and infected treatments.

Table IV-4. ANOVA results for plasma lysozyme and chitinase activities.

Two-Way Analysis of Variance (Unweighted Means Analysis)						
Dependent Variable		Factor 1		Factor 2		
Plasma Protein		Treatment		Day		
		Fixed Levels		Fixed Levels		
Source	<i>Sum of Squares</i>	<i>df</i>	<i>Mean Square</i>	<i>F</i>	<i>P > F</i>	<i>Omega²</i>
Treatment	192.875	2	96.4374	5.38389	0.013	0.00548906
Day	176.157	1	176.157	9.83449	0.005	0.00553082
Interaction	187.569	2	93.7845	5.23579	0.015	0.00530362
Error	358.244	20	17.9122			
Total		25				
Combined Effects:						0.0163235

Two-Way Analysis of Variance (Unweighted Means Analysis)						
Dependent Variable		Factor 1		Factor 2		
Lysosome Activity		Treatment		Day		
		Fixed Levels		Fixed Levels		
Source	<i>Sum of Squares</i>	<i>df</i>	<i>Mean Square</i>	<i>F</i>	<i>P > F</i>	<i>Omega²</i>
Treatment	0.00375183	2	0.00187591	0.145292	> 0.5	0
Day	0.00325511	1	0.00325511	0.252112	> 0.5	0
Interaction	0.0535058	2	0.0267529	2.07204	0.152	0.00712194
Error	0.258227	20	0.0129114			
Total		25				
Combined Effects:						0

Two-Way Analysis of Variance (Unweighted Means Analysis)						
Dependent Variable		Factor 1		Factor 2		
Chitinase Activity		Treatment		Day		
		Fixed Levels		Fixed Levels		
Source	<i>Sum of Squares</i>	<i>df</i>	<i>Mean Square</i>	<i>F</i>	<i>P > F</i>	<i>Omega²</i>
Treatment	3.69771	2	1.84885	10.6283	< 0.001	0.10044
Day	0.788511	1	0.788511	4.53281	0.046	0.0184268
Interaction	2.14165	2	1.07082	6.15571	0.008	0.0537835
Error	3.47913	20	0.173956			
Total		25				
Combined Effects:						0.172651

Examination of the tissue (Spleen, Head Kidney, and Kidney) lysozyme and chitinase activities at Day 2 (Table IV-5) and Day 8 (Table IV-6) after treatment found no significant effect of treatment. The only significant difference was the higher tissue chitinase activity found at Day 8 in kidney tissue.

Table IV-5.ANOVA results for tissue lysozyme and chitinase activities at Day 2.

Two-Way Analysis of Variance						
Dependent Variable		Factor 1		Factor 2		
Lysosome Activity		Treatment		Tissue		
		Fixed Levels		Fixed Levels		
Source	<i>Sum of Squares</i>	<i>df</i>	<i>Mean Square</i>	<i>F</i>	<i>P > F</i>	<i>Omega²</i>
Treatment	0.451856	2	0.225928	0.241144	> 0.5	0
Tissue	17.03	2	8.51501	9.08849	0.002	0.377437
Interaction	4.87263	4	1.21816	1.3002	0.307	0.0280167
Error	16.8642	18	0.936901			
Total	39.2187	26	1.50841			
Combined Effects:						0.370043

Two-Way Analysis of Variance						
Dependent Variable		Factor 1		Factor 2		
Chitinase Activity		Treatment		Tissue		
		Fixed Levels		Fixed Levels		
Source	<i>Sum of Squares</i>	<i>df</i>	<i>Mean Square</i>	<i>F</i>	<i>P > F</i>	<i>Omega²</i>
Treatment	5.21312	2	2.60656	0.576314	> 0.5	0
Tissue	21.6862	2	10.8431	2.39742	0.119	0.096016
Interaction	18.8175	4	4.70437	1.04014	0.414	0.0055163
Error	81.4107	18	4.52281			
Total	127.127	26	4.88952			
Combined Effects:						0.072421

Table IV-6.ANOVA results for tissue lysozyme and chitinase activities at Day 8.

Two-Way Analysis of Variance						
Dependent Variable		Factor 1		Factor 2		
Lysosome Activity		Treatment		Tissue		
		Fixed Levels		Fixed Levels		
Source	<i>Sum of Squares</i>	<i>df</i>	<i>Mean Square</i>	<i>F</i>	<i>P > F</i>	<i>Omega²</i>
Treatment	0.35387	2	0.176935	0.642511	> 0.5	0
Tissue	0.362151	2	0.181075	0.657545	> 0.5	0
Interaction	1.38562	4	0.346405	1.25791	0.306	0.0247803
Error	9.08756	33	0.275381			
Total	11.1892	41	0.272907			
Combined Effects:						0

Two-Way Analysis of Variance						
Dependent Variable		Factor 1		Factor 2		
Chitinase Activity		Treatment		Tissue		
		Fixed Levels		Fixed Levels		
Source	<i>Sum of Squares</i>	<i>df</i>	<i>Mean Square</i>	<i>F</i>	<i>P > F</i>	<i>Omega²</i>
Treatment	7.72711	2	3.86355	1.85093	0.173	0.0184576
Tissue	76.8035	2	38.4018	18.3973	< 0.001	0.377366
Interaction	36.9613	4	9.24034	4.4268	0.006	0.148662
Error	68.8829	33	2.08736			
Total	190.375	41	4.64329			
Combined Effects:						0.544486

IV.C.2. *In vitro* Onmy-Chit.02 and TNF expression levels in adherent cells

Based on the *in vivo* studies described above it appears that wounding can elevate plasma levels of chitinase activity. It is unclear from this study whether there was an increase in expression per cell or whether there was simply more cells expressing chitinase which gave rise to the increase in the plasma. In order to address this question I turned my focus to the response head kidney adherent cells would show when treated with various known stimulators of macrophages.

In a preliminary trial to determine the time course for exposure I observed that both chitin and LPS could induce TNF (tumor necrosis factor) expression in adherent cells by 24 hours post exposure. However, no response was observed in Onmy-Chit.02 expression at any time point. Shibata et al. reported that only chitin particles of 1-10 μm diameter are able to activate macrophages in mice. The chitin particle I used in the preliminary trial was larger than this threshold. Therefore, in the second trial chitin particles between 1-10 μm were used.

For the second trial I decided to use absolute quantification of transcript abundance. Plasmid DNAs containing Onmy-Chit.02 and TNF templates were used to construct absolute standard curves. Based on a standardized input of 25 ng of input mRNA I quantified in pg the transcript abundance of Onmy-Chit.02 and TNF. The data are summarized in Figure IV-5. Onmy-Chit.02 expression level variation range in Control groups from 4.9pg to 8pg per 25 ng of mRNA; chitosan from 5.8 to 6.3; chitin from 5.2 to 7.6; β -glucan from 4.5 to 10.2; LPS from 4.0 to 9.9; and zymosan from 1.8 to 7.9. Only in the 8 hr sampling point, was Onmy-Chit.02 expression level in both β -glucan and

LPS treatments significantly higher than control (t-test $P < 0.05$). A close observation of the data in Figure IV-5 shows that zymosan exposure may down regulate expression of Onmy-Chit.02. There is no evidence that chitin or chitosan particles are able to induce Onmy-Chit.02 expression

As with my earlier trial TNF mRNA expression reaches the highest expression level at 16hr after LPS stimulation. Besides LPS, β -glucan was also able to induce TNF expression but not as greatly as LPS. However, contrary to preliminary trial, chitin was not able to induce TNF expression. This discrepancy could be due to particle size difference in the two experiments.

IV.D. Discussion

The work of G. J. H. Lindsay provides an in-depth examination of gastric chitinase expression in fish, in particular, on chitinases of the rainbow trout (*Onchorynchus mykiss*, formerly *Salmo gairdneri*). Rainbow trout are an excellent species to use as a model because they are insectivorous throughout their lifecycle, relying on chitinous invertebrates for most of their diet. Furthermore, a survey of chitinase activity in the stomachs of north European fish (29 species) showed that rainbow trout have among the highest gastric chitinase activities[87]. This study found no correlation between gastric chitinase specific activity and the amount of chitin consumed by the species. However, there was a tenuous correlation between chitinase activity and the method of consuming prey: those species that mechanically disrupt their prey had low gastric chitinase activities (e.g., cyprinids have pharyngeal teeth; wrasse and spotted ray have a modified buccal cavity; grey mullet has a gizzard; tilapia use teeth to disrupt prey). Those that gulped their prey whole (Salmonids, Gadoids, perch, eel, red mullet, gurnards, mackerel) had high activity. It was inferred from this pattern that gastric chitinase in fish may function in disrupting the prey items, exposing soft inner tissues to the digestive juices, rather than functioning directly in digestion. Lindsay [87]also hypothesized that gastric chitinases may function in preventing blockage in the gastro-intestinal tract by awkwardly shaped chitinous structures; gut blockage is a common cause of mortality, especially in young fish.

Lindsay [122]reported that gastric chitinase activity is not affected by the presence of cellulose or polyethylene, but is reduced by 90% in the presence of dietary chitin. This is presumably because the enzyme is interacting with, but not necessarily

cleaving, the substrate. It was also found that gastric lysozyme readily adsorbs onto both chitin and cellulose, concluding that chitin in the diet may actually interfere with lysozyme function (typically considered to be defense against bacterial invasion).

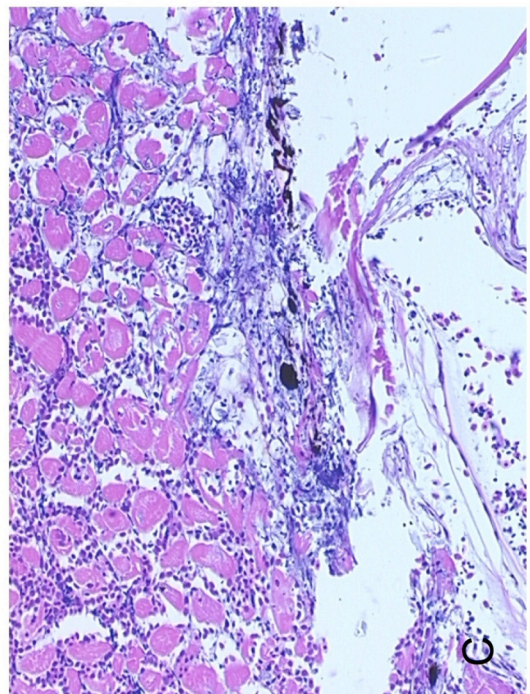
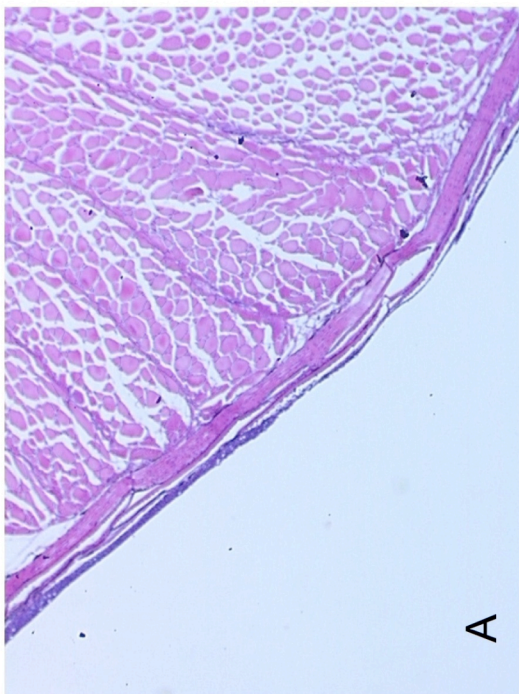
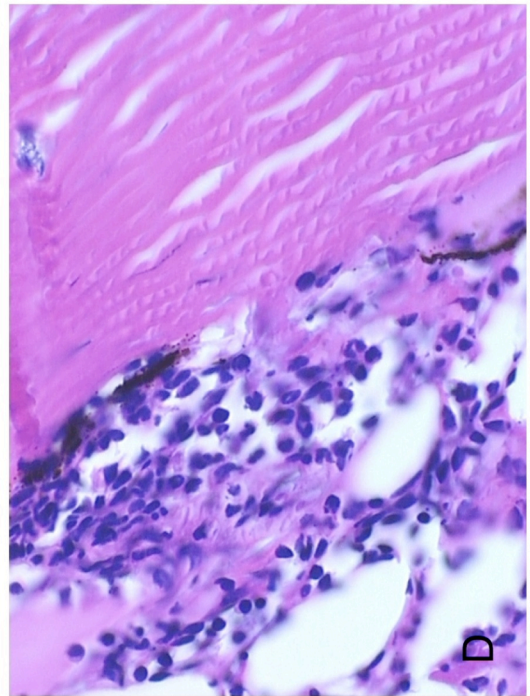
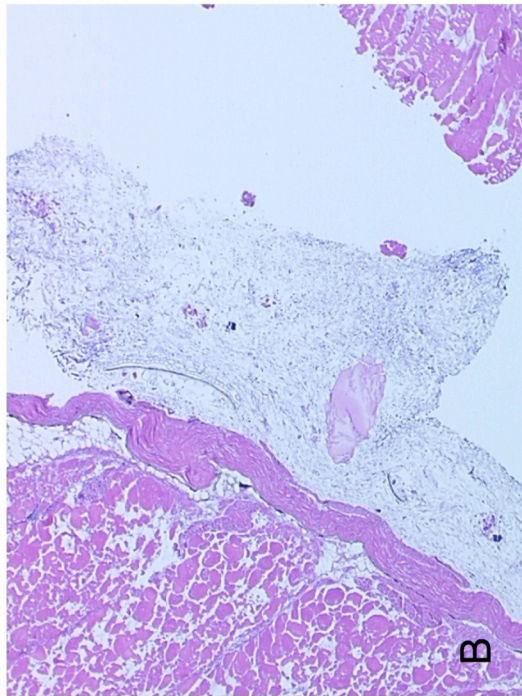
Lindsay reported on the production of chitinase activity in the developmental stages in rainbow trout [85]. After larval trout emerge from the egg they are called yolk-sac-fry because of the attached yolk which supplies them with nutrition for the first few days and weeks of life. During this time, the larvae do not have a well developed gut and do not feed. All their nutrition comes from the yolk sac. In this study, chitinase was first detectable in the yolk sac late stage and then increased rapidly until reaching its maximum at day 50 after hatching. This level was not significantly different from the adult enzymatic activity level. These data concur with a study of the development of protease, maltase and amylase in rainbow trout [179]. The ontogeny of chitinase activity in fish was studied in red sea bream. Chitinase was detected in eggs of red sea bream and chitinase activity (activity per gram of larva) increased proportionally throughout development of the larvae until the initiation of feeding, when it rose rapidly[94]. Lindsay [85] showed that chitin is not needed to trigger the production of gastric chitinase, since these larval fish were not fed a diet containing chitin. Based on this study, the gastric chitinase of rainbow trout can be considered constitutive.

Growth and digestibility studies of rainbow trout have shown a significant reduction in growth of rainbow trout fed diets containing 4, 10 and 25% chitin over a 12 week period[122]. After the initial growth experiment, stomachs were tested for chitinase activity. No difference was found between fish fed varying amounts of chitin. Addition of an antibiotic (Tribrissen®) to the diet, although greatly reducing the gut microflora, did

not change the chitinase activity of the stomach. Furthermore, addition of live chitinolytic bacteria (*Vibrio alginolyticus*) did not increase enzyme activity. Previous scanning electron microscopy studies[180] reported that trout stomachs do not have bacteria attached to the mucous membrane of the stomach. Lindsay hypothesizes that the harsh physiochemical conditions of the stomachs of trout prevent bacterial colonization. He concludes that the primary gastric chitinolytic activity in rainbow trout, and perhaps all fish, is endogenous. Also in this study, it was shown that the digestibility of chitin (by the chromic oxide method) is only about 3% and not significantly different than zero. In comparison, digestibility of lipids is 95%, proteins over 85%, and polysaccharides 20-25%.

The data presented in this thesis clearly shows that chitinase is endogenous to rainbow trout having established that there are two genes coding for this enzyme. The data presented in the current study indicates that chitinase expression as evidenced by increases in enzymatic activity in the plasma responds to wounding. Moreover, the level of Onmy-Chit.02 transcript in adherent macrophages appears not to be inducible by agents shown to induce TNF expression. The endogenous transcript levels in adherent macrophages are above the level obtained by TNF under heavy activation. I infer from these studies that Onmy-Chit.02 is constitutively expressed in mature activated macrophages.

Figure IV-1. Hematoxylin&Eosin staining of abraded and infected trout muscle. A) Control; B) Fungal infection growing on the abraded muscle tissue; C) Inflamed muscle tissue; D) 400X magnification of inflamed tissue showing infiltration by macrophages.



Plasma Chitinase Activity

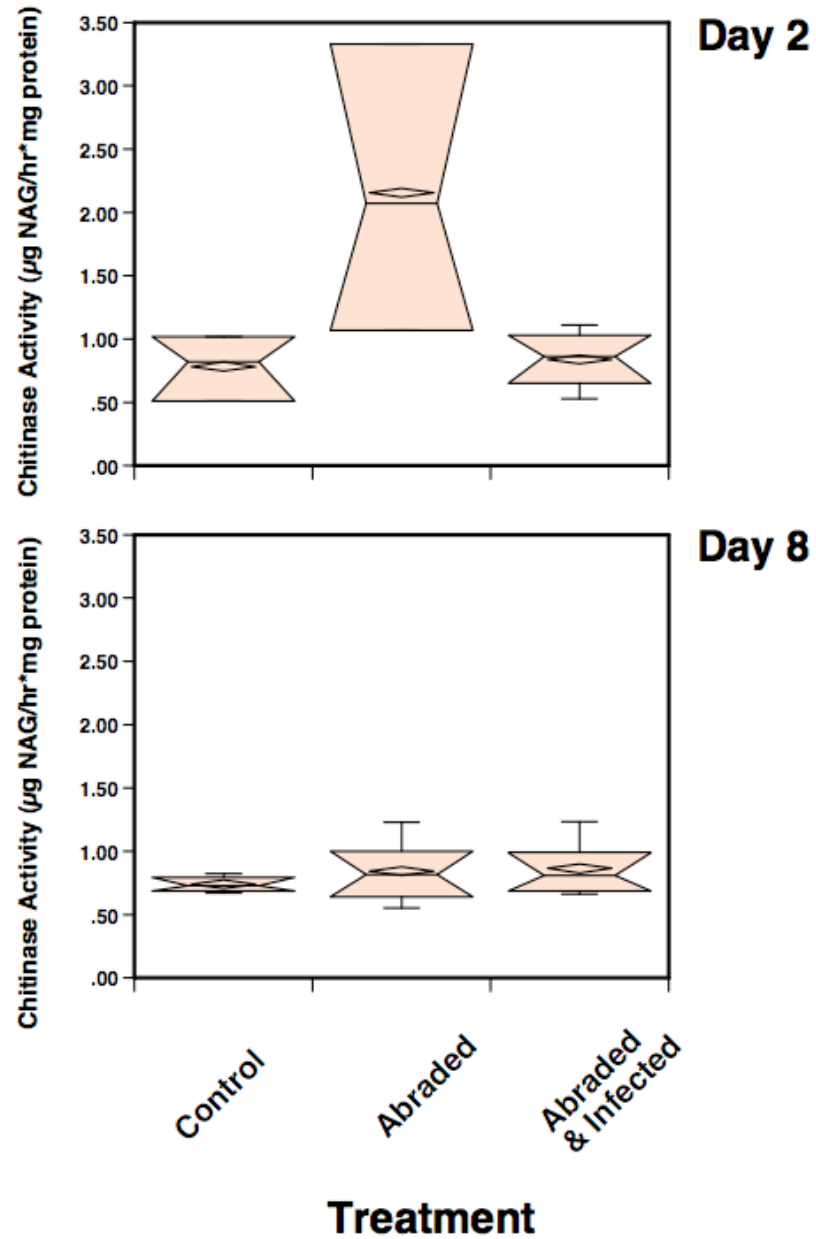


Figure IV-2. . Bar whiskers plots of plasma chitinase activity ($\mu\text{g NAG/hr} \cdot \text{mg protein}$) on Day 2 and Day 8 after treatments. The star indicates the mean value of the replicates. $N = 3$ for Day 2 and $N = 5$ for Day 8.

Tissue Chitinase Activity

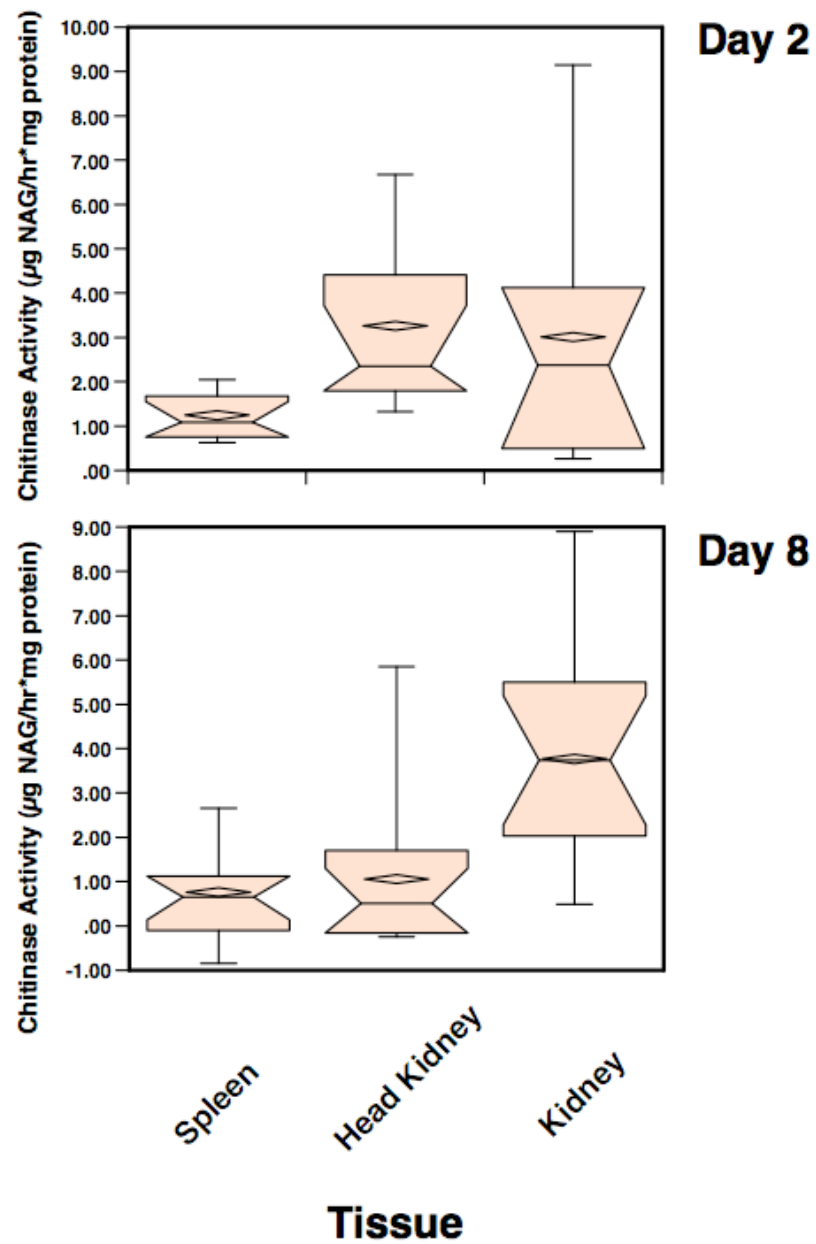


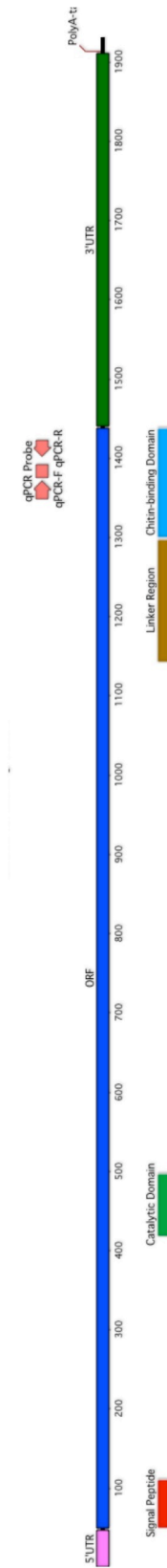
Figure IV-3. Bar whiskers plots of tissue chitinase activity ($\mu\text{g NAG/hr} \cdot \text{mg protein}$) on Day 2 and Day 8 after treatments. The star indicates the mean value of the replicates. $N=3$ for Day 2 and $N=5$ for Day 8.

Figure IV-4. Schematic showing the location of the qPCR primers and probes used for measuring transcript abundance of

A. Onmy-Chit.02

B. TNF

A



B

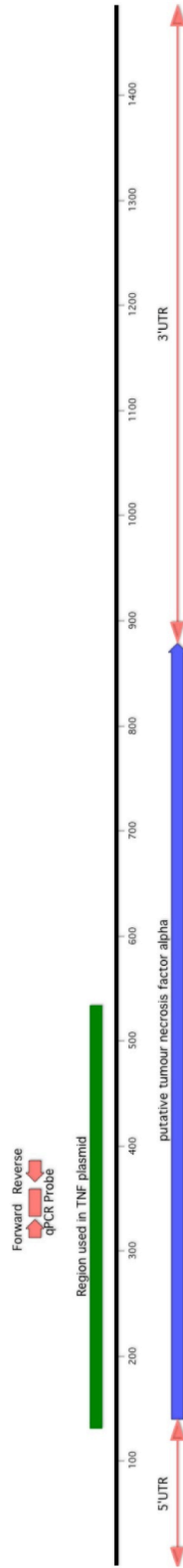
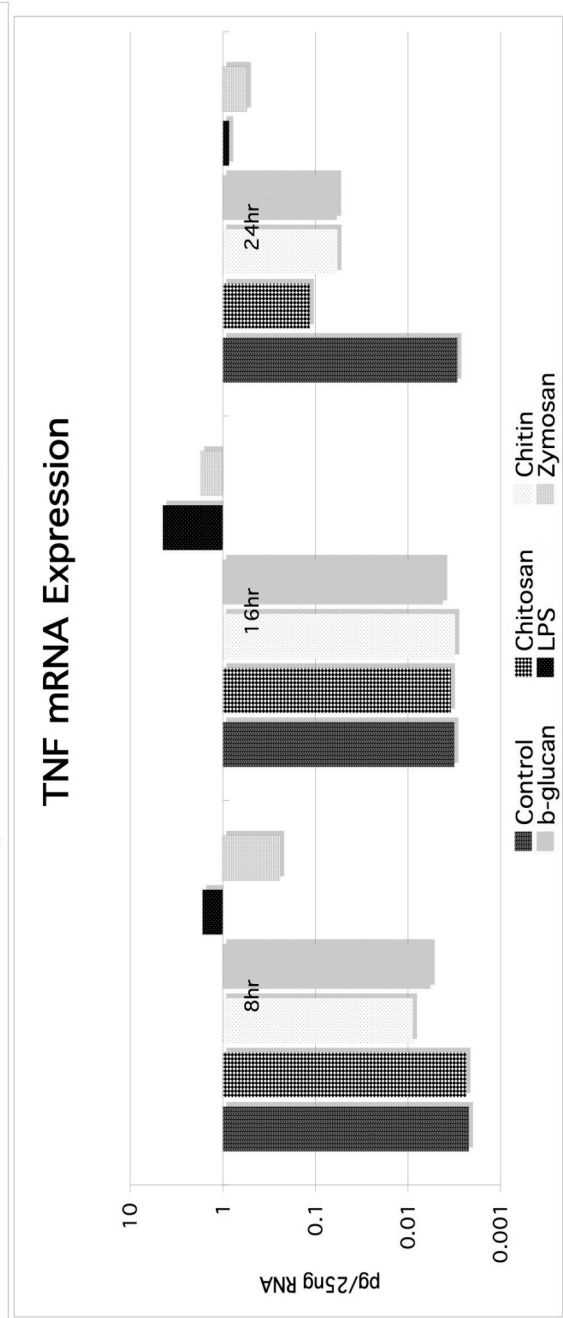
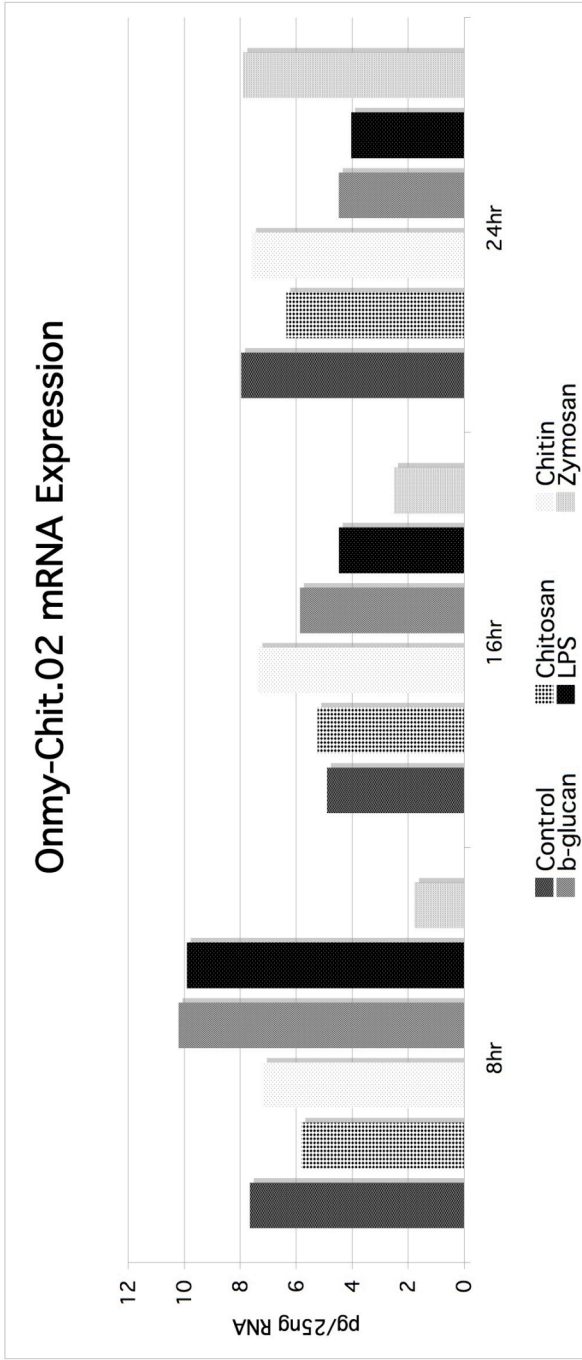


Figure IV-5. qPCR measurements of Onmy-Chit.02 and TNF transcripts in adherent macrophages of rainbow trout. Absolute transcript abundance per 25 ng of mRNA input is presented for cell s treated with designated agents.



Chapter V General Discussion and Future Research Direction

Traditional vertebrate physiology asserts that humans, fish and other vertebrates do not possess endogenous chitin. When vertebrate chitinases have been observed, it has been assumed that they were operating on exogenous substrates provided by the diet. Generally in vertebrates, the highest chitinase activities are observed in gastric tissues and the anecdotal link to chitin in the diets leads to an easy and satisfying role for the enzyme to be involved in digestion.

The origin of vertebrate stomach starts with the gnathostomes[181]. Indeed, most of the typical features of what is generally thought of as the vertebrate body plan (including stomach) are gnathostomes (jawed fish) characters. It is believed that a whole genome duplication happened between jawless and jawed fish[181]. There is no direct experimental proof for this since all the transistional species originated in this evolutionary period went extinct. The origin of vertebrate stomach also happened in this evolutionary period. The jawless fish -hagfish and lamprey lack a stomach. But most of members of jawed fish, both in the chondrichthyes and osteichthyes, do possess a stomach within the foregut. In the stomachless vertebrate, the esophagus empties its contents directly into the small intestine where nutrients from small food particles are absorbed. Because these animals do not have a storage receptacle for ingested food, they must continuously feed[181]. But in fishes with stomach, food can be temporally stored in foregut.

In Chapter II I describe that Onmy-Chit.01 is predominately expressed in the stomach, with high mRNA expression in the gastric gland. Its protein secretes along mucosal folds to the stomach lumen. Its expression pattern is identical to pepsinogen,

which is one of the major aspartic acid proteases secreted in the stomach. Furthermore, our lab also characterized that Onmy-Chit.01 is an exochitinase, which releases NAG dimers from non-reducing end of the chitin polymer. Needing to cleave a substrate from one end doesn't appear to be very efficient trait for a digestive enzyme to work. An endo-chitinase that hydrolyzes the chitin fiber at any point in the chain would have many more advantages. However, if the native substrate is short with many ends, an exochitinase could be quite successful *in vivo*. Studies of the yeast cell wall structure does show that a chitin oligomer is attached to an anchor polymer (a glycosylphosphatidylinositol component) with the non-reducing end facing out [182]. Thus, the fungal chitin found in the cell wall would be an ideal substrate for Onmy-Chit.01.

Cold-water fish, like rainbow trout, do not have a long digestive tract and hence food sits in the stomach longer than an equivalently sized homeotherm. In addition, its stomach pH is higher than mammals, usually around pH 4.5 compared to pH 2. All these conditions gave pathogens, such as fungi, a better chance to survive in the stomach. I would like to hypothesize that an ectotherms strategy on eliminating these pathogens could incorporate gastric chitinase. Similar to arguments presented for ruminant lysozymes, gastrointestinal chitinases might have a dual function in defense and food processing. Besides digesting dietary chitin for nutritional purposes, gastric chitinase could break down the tough chitin elements of pathogens and inhibit hyphal fungal growth.

I have shown that Onmy-Chit.02 is expressed in immunity related organs, primarily in the myeloid cell lineages. In Chapter IV, I demonstrated that under my experimental conditions, Chit.02 was not induced by exogenous chitin or zymosan. It is likely that

Chit.02 is constitutively expressed in some myeloid cell lineages. An increase in chitinase activity in a tissue could be achieved by influx of myeloid cells, such as macrophages, now expressing chitinase. In Chapter III, I discovered that IRF-2 may play a role in Onmy-Chit.02 regulation after comparing TFBSs (transcription factor binding sites) of the three rainbow trout chitinase genes. In future experiments, IFN- γ and TNF- α etc. cytokines should be included in stimulants. In human, IFN- γ , TNF- α and LPS promote chitotriosidase gene expression in macrophages[148]. Since it is known that macrophages treated with IFN- γ and TNF- α develop an increased cytotoxic activity against intracellular microorganisms and tumor cells, it is very likely that chitotriosidase is involved in a cellular response elicited by this regulatory cytokine[148].

Based on the phylogenetic analysis (Figure II-13), Onmy-Chit.02 is more closely related to a primitive source. In invertebrates, chitinase is primarily involved in tissue remodeling and molting, which means the chitinase works on the endogenous chitin. On the contrary, in vertebrates, chitinase appears to work primarily on exogenous sources of chitin. This discrepancy may lead to the complete change on regulatory regions of chitinase.

Even though a chitin long chain polymer has not been found in vertebrates, chitin oligomers (less than nine NAG) have been detected in fish and frog. It has been proposed that the vertebrate endogenous chitin-oligomer is primarily involved in embryonic development[183-187]. When chitinase activity was inhibited by allosamine (a specific chitinase inhibitor) in zebrafish embryo, the whole embryo showed gross deformities. This role of chitinase in tissue remodeling may not be distinct from an anti-fungal role. *Ichthyosporidium* sp. is a common fungal pathogen of fish. Fish connective tissue cells

respond to invasion of this pathogen by proliferating and encapsulating the pathogen in a fibrous cyst [188] – by reorganizing its tissues. Perhaps the anti-fungal nature of chitinases is an offshoot of an ancestral tissue-remodeling role.

I hypothesize that duplication of the chitinase locus happened twice in vertebrate evolution. The first time was between jawless fish and jawed fish, a period believed to correspond to whole genome duplication in bony fish. The primordial immunity related chitinase locus duplicated into two loci, one of them kept for immune function while the other one was adapted to be expressed in the gastric system. This gastric chitinase would play a dual role in both food processing and anti-pathogen protection. Both roles co-evolved with stomach genesis. While the gastric chitinase may not exist in some of stomachless animals, such as zebrafish, fugu etc, this is a loss of function. I predict that two chitinase genes will be found in cartilaginous fish since they are first order of gnathostomes and most of species possess a stomach. While I did find chitinolytic activity and cross-reacting material to our anti-Onmy-Chit.01 antibody in the stomach of Atlantic stingray, nurse shark and sturgeon, I do not know which form it is. In addition, chitinolytic activity also had been found in the plasma of *Etmopterus spinax* (velvet belly shark) and *Raja radiata* (Starry ray) [189].

The second duplication of chitinase could be species specific, such as in our model animal, rainbow trout. Trout and salmon are actually partial tetraploids, $2n = 4\phi$ [190, 191]. The term ‘partial’ means that the species have undergone an ancient extra whole genome duplication (i.e. in addition to the two rounds of whole genome duplication which occurred in the vertebrate lineage [13]), and are currently reverting to diploidy via a process of gene loss. I believe Onmy-Chit.01 was duplicated and underwent a

recombination which resulted in two copies on the same chromosome. This exchange could also be mediated by the extensive transposons observed in the rainbow trout genome. This duplication could happen in lungfish and amphibians[192]. This hypothesis was supported by the evidence that both mammalian chitotriosidase and AMCase have a chitinase-like protein close to them (Figure III-15&16). The chitinase-like protein kept the family 18 protein structures but exhibit no catalytic activity.

To answer the question whether Onmy-Chit.02 is primordial, I blasted rainbow trout chitinase sequences against *Ciona intestinalis* (sea squirt) [193] and *Branchiostoma floridae* (Amphioxus) [194] genomic database because *Ciona* is a basal urochordate and amphioxus is a primitive vertebrate. Both species contain chitinase based on my blast analysis. In both cases, the identity score to Onmy-Chit.02 is slightly higher than Onmy-Chit.01 but it is not significant enough for us to conclude that Onmy-Chit.02 or Onmy-Chit.01 is closer to them phylogenetically. Expression pattern studies are definitely needed to confirm the role for chitinase in these primitive chordates. The fact is that both species may contain multiple chitinases.

Table V-1. Clustal_W analysis result of four chitinases.

	Identity Scores (%)			
	Onmy_CHIT.02	Onmy-CHIT.01	<i>Ciona</i> chitinase	<i>Amphioxus</i> chitinase
Onmy_CHIT.02	100	56.2	11.2	10.1
Onmy-CHIT.01	70.1	100	8.4	9.2
<i>Ciona</i> chitinase	24.1	23.2	100	9.2
<i>Amphioxus</i> chitinase	23.2	24.2	24.1	100
	Similarity Scores (%)			

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